PB-227 571

HISTOCHEMICAL AND CYTOPHOTOMETRIC ASSAY OF ACID STRESS IN FRESHWATER FISH

Pennsylvania State University

Prepared for:

Environmental Protection Agency

May 1971

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HISTOCHEMICAL AND CYTOPHOTOMETRIC ASSAY OF ACID STRESS IN FRESHWATER FISH

by

The Pennsylvania State University
Department of Biology
University Park, Pennsylvania 16802

for the

ENVIRONMENTAL PROTECTION AGENCY

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Grant Number DXJ 18050

May 1971

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ABSTRACT

aminosalicylic acids

The feasibility of using histochemical and histopathological changes in brook trout, longnose dace and fathead minnows as bioindicators of acid pollution was investigated. Laboratory studies entailed using a gravity flow diluter system. Field studies involved using net traps in polluted streams. Exposure durations were 4-5 days and 28-30 days. Histochemical and cytophotometric analyses were made of gills, Stannius corpuscle, blood, spleen, kidney and liver.

The primary mode of acid toxicant action is gill damage which results in impaired respiratory, excretory and liver functions. Short term indices of acid stress include: colloidal iron and PAS staining of gills and renal Stannius corpuscles. A useful bioindicator of prolonged acid exposure is decreased azure B-RNA staining of liver cells; this assesses the extent of liver impairment and reflects a reduced tolerance of fish to other toxicants.

Sublethal levels of acidity are not cumulative. However, pH levels of about 5.0 should be considered hazardous since they prove toxic to breeding fish having increased oxygen needs and also reduce the liver's ability to detoxify noxious substances present in acid polluted waters.

This report was submitted in fulfillment of Grant No. 18050 DXJ under partial sponsorship of the Water Quality Office, Environmental Protection Agency.

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SECTION I

CONCLUSIONS

(A) General Conclusions (relating to utility of histochemical methodology)

1. A number of histochemical methods, primarily developed and used for diagnosis of human disease states, can be used to assess toxicant effects in fish of sublethal levels of water acidity under laboratory and field conditions.

2. Analytical histochemical techniques, which involve use of cytophotometry (microscopic microchemistry), enable the detection of tissue chemical changes in the picogram (10^{-12}g) range with a routine accuracy of ±2%. Thus, for microscopic analysis of organic constituents in tissue cells, the detection limit, sensitivity and accuracy of cytophotometry approach theoretical limits imposed by light microscopy.

3. Analytical histochemistry is a simpler and more versatile tool than most physical analytical procedures, in that -- purified samples are not required, minimal amounts of tissue (<1.0mg) are needed and routine processing, microtomy and staining methods are generally involved. Instrumentation is also not costly; microspectrophotometer components can be purchased for less than $5,000 if a laboratory is already equipped with a research microscope.

4. Use of a battery of several histochemical tests is preferable to a single test in establishing safe versus harmful levels of stream pollutants since measures based on combined tests enable one to differentiate between acclimative and debilitative responses.

(B) Specific Conclusions (regarding acid induced histochemical changes in fish)

5. Histochemical changes in mucopolysaccharides and nucleic acids of the trout gill and renal Stannius corpuscle cells are useful short-term (1-7d) indices of sublethal levels of water acidity. These are transient changes and are not evidenced in 28 day exposed fish.

6. Ribonucleic acid levels in certain renal blood cell precursors are depressed during acute exposure and elevated during prolonged (28 d) exposure of trout to sublethal levels of acidity and can thus serve as a useful index of acid pollution in both acute and subacute exposure test situations.

7. Hematocrit (or hemoglobin) measures, leucocyte differential counts and total serum lipid levels are generally more useful supplemental indices of hemal responses to sublethal levels of acid exposure than biochemical measures of blood pO_2, radioiron incorporation, radioiodinated serum albumin, electrophoretic histone analyses or chromatographic analyses of serum fatty acids.

8. A given level of water acidity is much more toxic under field conditions (i.e. in presence of other stream contaminants) than under controlled laboratory conditions.

9. Reproductively active trout are less tolerant to acid water than nonbreeding trout.

10. Brook trout, fathead minnows and longnose dace exhibit the same basic pattern of acid induced histochemical and histological responses in gills, hemopoietic organs and circulatory elements.

11. Liver function is impaired in acute and subacute acid exposed trout as reflected in a decreased azure B-RNA content of liver cell nuclei. Since a major function of the liver is removal of blood toxins any overall decrease in liver metabolism would reduce the tolerance of fish to toxicants present in acid polluted streams.

SECTION II

RECOMMENDATIONS

Recommendation 1. Since standards for water quality must ultimately be based on biological as well as physical and chemical criteria, more emphasis should be placed on developing sensitive bioassays of water pollution. Because many histochemical methods are extremely sensitive and quantifiable, greater use of cytophotometry as a bioassay approach is recommended. Metal ion toxication should be especially amenable to bioassay using histochemical techniques since, unlike acid pollution, effects of heavy metals tend to be cumulative.

Recommendation 2. Careful consideration should be given to concentrating attention on using fish tissue changes as biological indicators of water pollution. Fish constitute a valuable resource from the conservational, recreational and economic standpoint. Of equal importance, since fish and man exhibit remarkable similarities in metabolic regulatory mechanisms at the cellular level, pollutants causing histopathology in fish should be regarded as potential health hazards.

Recommendation 3. Special study is required of what constitute "norms" for various biochemical, physiological and histological parameters in various fish species. Present information is deficient with respect to normal levels of tissue constituents as well as microscopic and histochemical appearance of healthy fish. Of particular interest to fish pathologists and physiologists would be data on hemal, endocrine and excretory tissue parameters.

Recommendation 4. There is an urgent need for intensified research on toxicant effects of mixtures of water contaminants since the development of adequate standards of water quality ultimately will require detailed information on the mode of entry, mechanism of action, cumulative effects, etc., of a specified toxicant in the absence and presence of other toxicants.

Recommendation 5. Serious consideration needs to be given to devising a general procedure for interlaboratory exchange of tissue specimens for analysis, as well as of material which has been histochemically analyzed, to speed up standardization of various types of analytical procedures on a nationwide basis. Implementation of such an exchange could be initiated by setting up a repository at one of the Federal Water Quality Laboratories.

SECTION III

HISTOCHEMICAL ANALYSES OF GILLS AND STANNIUS CORPUSCLES IN ACID EXPOSED BROOK TROUT

Preliminary work in our laboratory showed that acute exposure of trout to severe acidity (pH 3.9) disrupts the cellular integrity of gill lamellae (Plonka and Neff, 1969) and also results in depressed body sodium levels (Packer and Dunson, 1970). Since both gills and renal Stannius corpuscles are involved in ion and water regulation (Conte, 1969; Fontaine, 1964), a series of experiments were undertaken to investigate the nature of histochemical responses in these organs in fish subjected to sublethal levels of acidity for short (4-5 day) and relatively long (28 day) durations of exposure. The specific aims of this phase of work were: (1) to analyze histochemical changes in gills and Stannius corpuscles with methods selected to provide measures of cellular constituents involved in regulation of metabolism (i.e., nuclear DNA, RNA, and histone content) as well as measures of cytoplasmic endproducts of synthesis (mucin, PAS positive material or nonspecific protein content); (2) to determine which tests show promise as potential short-term bioassays for toxicant effects of sublethal levels of water acidity; (3) to compare histochemical effects of subacute (28 day) versus acute (4-5 day) exposures to sublethal levels of acidity on designated parameters; and (4) to elucidate the physiological basis of histochemical response patterns in terms of possible long-term effects on the functional capacity of the gills and Stannius corpuscle.

Methods. Tissue specimens came from three sources: hatchery reared brook trout (*Salvelinus fontinalis*), obtained from the Pleasant Gap Fish Research Station, Pleasant Gap, Pennsylvania; wild brook trout, caught in Tomtit Run, Clearfield County, Pennsylvania; and fathead minnows (Pimephales) (fixed in 4% formalin, preserved in 70% ethanol) obtained from the National Water Quality Laboratory, Duluth, Minnesota. Prior to experimentation, trout were acclimated to laboratory conditions for one to two weeks in 150 gallon tanks at 12 C under constant aeration. At the end of each test run, all fish were sacrificed by a sharp blow on the head, weighed (±0.1 gm), measured from head to caudal fin (±0.5 gm), dissected, and sexed. Hatchery trout varied in length from 17-20 cm and weighed 75-120 gms with the exception of one group of larger fish used for an acute study (21-25 cm; 170-225 gms). Wild trout varied in length from 11-18 cm and weighed 10-60 gms.

Acute toxicity tests (4-5 days) were conducted in two systems. In one, a group of 50 control and 50 experimental hatchery reared trout were maintained in a static system. The water was not changed during the 4-5 day period, although it was constantly aerated, filtered and kept at 12 C. Acidity was maintained by addition of concentrated HCl (specific gravity 1.84; 37% by weight) to establish a pH of 4.0 ± 0.2. Control tanks were maintained at a pH of 7.0 ± 0.5. In the second system, 70 wild brook trout were exposed to varying levels of acidity in a gravity flow

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Table 1. Histochemistry of hatchery reared brook trout gill following acute (4-5 day) and subacute (28 day) exposure to acid water

N	Treatment	Staining reactions	No. of analyses[a] (No. of fish)
50	Control, pH 7.0-7.5 for 5 days	1. Colloidal iron, PAS, alcian blue and mucicarmine for mucopolysaccharides	300(100)
50	Acid exposed, pH 4.0 for 5 days	2. Feulgen cytophotometry for DNA	200(20)
		3. Eosin-fast green, alkaline fast green and eosin Y for histones	70(36)
		4. Gomori's trichrome and hematoxylin and eosin for histological detail	20(10) 100(50)
20	Control pH 7.0 for 28 days	1. As above	120(40)
		2. As above	200(20)
20	Acid exposed, pH 4.8 for 28 days	3. As above	20(10)
		4. As above	20(10) 40(20)

[a]The number of analyses refers to the number of tissue sections prepared and examined microscopically. Where cytophotometry was performed the number of analyses represents the number of individual cellular measures.

Table 2. Histochemistry of hatchery reared brook trout Stannius corpuscle following acute (4-5 day) and subacute (28 day) exposure to acid water

N	Treatment	Staining reactions	No. of analyses[a] (No. of fish)
50	Control, pH 7.0-7.5 for 5 days	1. Colloidal iron, alcian blue and mucicarmine for mucopolysaccharides	300(100)
		2. Periodic acid Schiff for aldehydes (cytophotometry)	200(50)
50	Acid exposed, pH 4.0 for 5 days	3. Feulgen cytophotometry for DNA	200(20)
		4. Azure B cytophotometry for RNA	180(20)
		5. Eosin-fast green, alkaline fast green and eosin Y for histones	20(10)
		6. Sudan black B for lipid	20(10)
		7. Gomori's trichrome and hematoxylin and eosin for histological detail	20(10) 100(50)
20	Control pH 7.0 for 28 days	1. As above	120(40)
		2. As above	200(20)
		3. As above	200(20)
20	Acid exposed, pH 4.8 for 28 days	4. As above	150(20)
		5. As above	40(20)
		6. As above	20(10)
		7. As above	40(20) 40(20)

[a]The number of analyses refers to the number of tissue sections prepared and examined microscopically. Where cytophotometry was performed the number of analyses represents the number of individual cellular measures.

proportional diluter system (Mount and Brungs, 1967) with aquaria maintained at pH's of 4.1 ± 0.4, 4.7 ± 0.5, 5.1 ± 0.4, 6.6 ± 0.3, and 7.1 ± 0.2. Temperature was maintained at 12 C using an automatic cooling unit and pH monitored by a multichannel recorder system.

Tables 1 and 2 list various histochemical tests performed on gill and Stannius corpuscle tissue from acute acid exposed hatchery trout and wild trout exposed to graded levels of acidity.

Subacute toxicity studies (28 day) on a group of 53 hatchery trout were all conducted in a gravity flow diluter system. The pH's of the three experimental tanks were 6.3 ± 0.1, 5.9 ± 0.4, and 4.8 ± 0.2, and the pH of the control tank was 7.0 ± 0.2.

Chronic exposure data were based on tissue from fathead minnows exposed to graded severities of acid for seven months (in a gravity flow diluter) at the Duluth Water Quality Laboratory (Table 3). These fish were fixed in formalin (4%) and preserved in 70% ethanol before shipment by air express to our laboratory.

Table 3. Histochemistry of gill mucopolysaccharides in fathead minnows exposed to graded severities of pH for seven months

N	Treatment	Staining reactions for all groups	No. of analyses[a] (No. of fish)
20	Controls (two groups at pH 7.0 and 7.4)	1. Colloidal iron, alcian blue and mucicarmine for mucopolysaccharides	134(67)
47	Experimentals (five groups at pH 6.5, 6.0, 5.5, 5.0, and 4.7)	2. Hematoxylin and eosin for histological detail	67(67)

[a] The number of analyses refers to the number of tissue sections prepared and examined microscopically. Where cytophotometry was performed the number of analyses represents the number of individual cellular measures.

Portions of gills and kidney (with Stannius corpuscles) were fixed in 4% neutral buffered formalin for 24 hours, dehydrated, cleared, paraffin embedded and sectioned at 6μ. Touch preparations of Stannius corpuscles were also made, some of which were fixed in CH_3OH while others were stained immediately for lipids. Formalin fixed touch preparations were used for azure B-RNA staining. Sections of Stannius corpuscles and gills were analyzed for DNA using the Feulgen reaction, basic fuchsin, CI 42500 and for mucopolysaccharides using periodic acid Schiff's (PAS) reagent (Humason, 1967). Touch preparations of Stannius corpuscles were treated with DNAase (Worthington Corp.) and stained by azure B for RNA.

Cytophotometric measurements were made of DNA, RNA and PAS positive material using a single beam microspectrophotometer using the two wavelength method of cytophotometry described by Patau (1952) and Ornstein (1952). For F-DNA and PAS the wavelengths chosen were 560 nm and 495 nm; for azure B-RNA the wavelengths selected were 560 nm and 510 nm.

Nonhomogeneous areas (i.e., whole nuclei or entire cells) were measured by centering the nucleus in the photometric field, excluding as much surrounding light as possible.

Qualitative histochemical analysis of histones in gills and Stannius corpuscles were made using eosin Y-fast green (eosin Y CI 45380, fast green FCF CI 42053) (Bloch, 1966), alkaline fast green (Humason, 1967) and eosin Y (Bloch and Hew, 1966). Unfixed Stannius corpuscle touch preparations were also stained with Sudan black B, CI 26150 (Humason, 1967) for lipids. In addition, three mucopolysaccharide stains, colloidal iron, alcian blue, CI 74240 (Mowry, 1963) and mucicarmine, CI 75470 (Humason, 1967) were used for studies of mucin content in gill and selected Stannius corpuscle sections. For routine microscopic analyses, henatoxylin and eosin and Gomori's trichrome (chromotrope CI 16570; fast green FCF CI 42053) were employed (Humason, 1967) (Table 4).

Table 4. Chemical basis for histochemical and histological stains employed

Tissue substrate tested	Chemical groups in cells involved in staining	Reaction or stain employed
DNA	aldehydes	Feulgen reaction (Schiff reagent)
RNA	phosphates	basophilia (azure B and DNAase)
Nuclear proteins (histones)	basic amino acids	acidophilia (eosin-fast green)
Cytoplasmic lipids	lipids	mutual solubility of fat and dye (Sudan black B)
Mucopolysaccharides	acidic groups of mucopolysaccharides	(colloidal iron) chelate with metal ion subsequently reduced by K ferrocyanide
Mucopolysaccharides	carboxyl groups and sulfates	basophilia (alcian blue)
Mucopolysaccharides	unknown	mucicarmine affinity
Compounds containing free aldehyde groups	aldehydes	PAS reaction (Schiff reagent)
Acidophilic and basophilic constituents	anionic and cationic components	hematoxylin and eosin
Acidophilic and basophilic constituents	anionic and cationic components	Gomori's trichrome

A preliminary aspect of work involved a comparative evaluation of acid induced histochemical changes in gills and Stannius corpuscles of wild and hatchery reared trout. That differences between wild and hatchery bred trout exist in size, physical state, behavioral activity, and tolerance to water toxicants is well recognized. However, not unexpectedly, there were no differences in the histological or histochemical appearance of tissues or organs (including the gills and Stannius corpuscles) between fish obtained from the hatchery and those collected in field streams. Moreover, no differences were found in the type or intensity of acute acid induced histochemical changes which could be related to size, weight, sex or collection site of the fish used. Therefore, the following data are presented without reference to the source of fish.

General behavioral observations were made on fish exposed to acid water (pH 4.0-4.85). Both acute (4-5 day) and subacute (28 day) exposed trout exhibited a noticeable decrease in activity. Fish in acidic water ate poorly, whereas controls consumed 4-5 fish pellets per day. Wild trout, which were initially highly colored, showed progressive loss of coloration in all gradations of acidity (pH 4.5, 5.0, 5.5, 6.0) but blanching was most marked at low pH's (4.5, 5.0). Both subacute and acute acid exposed fish surfaced more than controls and gulped air through their mouths. In extremely low pH water (below 4.0) trout secreted copious amounts of mucus such that a mucous film became visible over their whole body, especially in the gill area. Fish in this state showed a loss of equilibrium, often swimming with their ventral sides up. Acute and subacute acid exposed trout also exhibited hyperventilation as reflected in an increased rate of opercular movements. No attempt was made to undertake a systematic study of behavioral changes. The intent was simply to confirm that exposure of trout to moderate and high levels of acidity did evoke external behavioral symptoms of respiratory distress. It was established, however, that behavioral reactions were most pronounced in the first four days and tended to diminish with continued exposure. The average mortality rate of subacute exposed trout maintained in control (pH 7.0) and moderately acid (pH 5.5, 6.0) aquaria was 10% whereas mortality at lower pH levels (pH 5.0, 4.5) was 30%.

Table 5. Mucous cell changes in acid exposed fish

N	Treatment	Presence of mucous cells	Location of mucous cells	Extracellular material	Filament clubbing
50	Hatchery trout, control, pH 7.0-7.5 for 4-5 days	+	- tip of filaments - base of lamellae	- to +	-
50	Hatchery trout, acute exposed, pH 3.7-4.0 for 4-5 days	+++ to ++++	- tip of filaments - along length of lamellae	+++ to ++++	+++ to ++++
5	Wild trout, control, pH 7.0-7.5 for 4-5 days	+	- tip of filaments - most at lamellar base, some along side	+	+
5	Wild trout, acute exposed, pH 4.0-4.5 for 4-5 days	+++ to ++++	- along lamellae - tip of filaments - between lamellae	+++	+++
20	Hatchery trout, control, pH 7.0-7.5 for 28 days	+	- tip of filaments - at lamellar base	- to +	-
20	Hatchery trout, subacute exposed, pH 4.0-4.5 for 28 days	++	- tip of filaments - along lamellae	++	++
67	Fathead minnows, chronic exposed, all gradations, pH 4.5-7.0 for 7 months	+	- at lamellar base[a]	-	-

[a] These observations were made from cross sections of whole fish; each filament was therefore cut and it was not possible to examine the tips of the filaments for mucous cells.

Microscopic analyses of gill changes in acute and subacute acid exposed brook trout are summarized in Table 5. A marked increase in the number of gill mucous epithelial cells was evidenced in acute acid exposed trout as well as in unattached epithelial cells, extracellular material and mucin so that interlamellar spaces were almostly completely obscured. In subacute acid exposure studies, trout exhibited a moderate increase in lamellar mucous cells and accumulation of extracellular materials, but the spaces between lamellae were not completely obliterated. Moreover, the distribution of mucous cells along the lamellae differed in control and subacute or acute acid exposed fish. Mucous cells of controls were almost exclusively limited to the base of each lamella whereas they were found along the entire lamellar length in acid exposed fish. Chronic exposed fathead minnows exhibited none of the changes observed in trout. There was no difference in relative numbers of lamellar mucous cells between acid exposed fish and controls and there was no evidence of increased cellular and extracellular material between lamellae.

In brief, the major findings in this phase of work can be summarized as follows. First, acute acid exposure elicited a marked increase in mucopolysaccharide staining of gill epithelial cells. This was more pronounced at the lowest pH levels (pH 4.0-5.0). Furthermore, both hatchery and wild trout exhibited the same intensity of mucification and extent of histopathology (interlamellar cell debris). Second, there appears to be a diminution in this response with increased duration of exposure. After 28 days of continuous exposure in low pH aquaria (pH 4.0-4.5), only a moderate increase in mucopolysaccharide staining was evidenced in trout gills while subacute exposures to moderate levels of acidity elicited little or no response. Significantly, there was no difference in the histochemical appearance of gills from fathead minnows exposed to pH gradations ranging from 4.5 to 7.0 after seven months of exposure.

Cytophotometric analysis of DNA in gill cells. Data summarized in Figure 1 represent a total of 400 individual measurements of nuclear Feulgen DNA (F-DNA). Determinations were made on gill supporting cells which are located at the lamellar base and can be distinguished from other gill epithelial cells by their oval shape and large vesicular nuclei. Because the increase in interlamellar material begins at the lamellar base and supporting cells are consistently found in conjunction with lamellar "clubbing," it was reasoned that nuclei of supporting cells might reflect either signs of damage (karyolysis) or changes in mitotic (i.e., proliferative) activity.

F-DNA data are conventionally presented as frequency histograms because this facilitates the detection of changes in the proportion of nuclei which exhibit either chromatin loss (reduced F-DNA content) or mitotic activity (tetraploid F-DNA level). To facilitate comparison of the exposure groups, dotted lines were included on histograms to designate the 2C DNA interval; the 2C level represents the nonreplicating diploid content of DNA. To insure that the value assigned as the 2C level of DNA truly represented the diploid amount of DNA, F-DNA content was also measured in 50 sperm cells from these fish. As expected, the mean DNA content for the sperm cells was one-half that of the somatic cells.

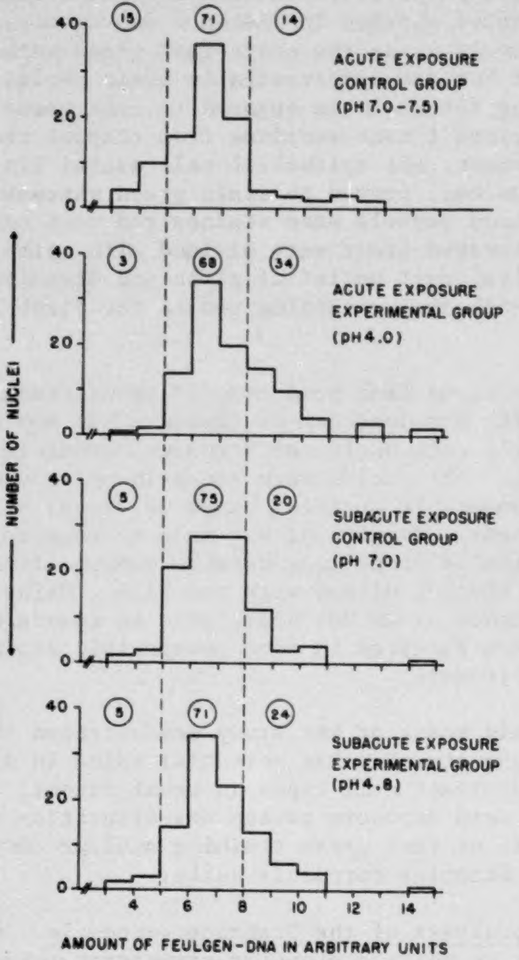

Figure 1. DNA distribution patterns for gill supporting cells of brook trout exposed to acid water for acute (4-5 day) and subacute periods (28 day). Numbers in circled inserts refer to percentage of cells between and on each side of dotted division lines.

As is evident from the data presented, there are few nuclei with 4C amounts of DNA, indicating that neither acute nor subacute acid exposure causes any stimulation in mitotic activity of supporting cell elements. Moreover, there is no appreciable increase in nuclei with very low DNA levels, indicating the absence of nuclear damage in acid exposed gills. Thus, supporting gill lamellar cells are not the major source of cellular debris which comprises the interlamellar coagulum present in acute acid exposed gills.

Histochemical analysis of basic nuclear proteins in gill cells. Another useful parameter of nuclear function is the histone or basic protein content. Relative changes in histones can be analyzed qualitatively or semiquantitatively using the eosin-fast green method providing there is assurance that one can differentially stain nuclei which exhibit preferential staining for eosin as opposed to fast green. Analyses of eosin-fast green stained tissue sections from control trout showed this to be the case. However, all epithelial cell nuclei (in both gills and Stannius corpuslces) proved to stain green whereas erythrocyte nuclei in adjacent blood vessels were stained red with eosin. When tissue sections from untreated trout were stained with either eosin Y or fast green, epithelial cell nuclei of gills and Stannius corpuscle showed an affinity for both dyes, staining red in the first instance and green in the second.

Analyses of sections from acid exposed trout revealed that neither the level of acidity nor duration of exposure had any effect on the intensity or color of gill cell nuclei or Stannius corpuscle nuclei with eosin-fast green staining. All nuclei were green in color and cells of acid exposed fish showed comparable staining based on visual examination of the stained specimens. No attempt was made to measure dye concentration cytophotometrically since considerable nonspecific staining occurred. For example, when control slides were run (i.e., using acetic anhydride to acetylate histones or 1N HCl hydrolysis to remove histones), both eosin Y and fast green resulted in some nonspecific staining of nonhistone cellular constituents.

In summary, this phase of the study demonstrated the following: First, eosin-fast green staining has potential value in differentiating between functionally distinct cell types in trout tissue. Second, there is no evidence that acid exposure causes any alteration in the relative amounts of eosinophilic or fast green staining nuclear components in either gill epithelial or Stannius corpuscle cells.

Histological analyses of the Stannius corpuscle. Grossly, Stannius corpuscles appear as whitish globular structures embedded in the middle third of the kidney. These vary in size from less than one to five millimeters in diameter, depending somewhat on the size of the fish. The number of Stannius corpuscles per trout range from 4 to 10; most trout had 3-4 large (3-4 mm) and 1-2 small (less than one mm) corpuscles; however, in some, no large and 8-10 small corpuscles were found. Thus, it would be very difficult to measure changes in the total mass of glandular elements (e.g., hypotrophic or hypertrophic responses) on a tissue area or on a weight basis.

Histologically, the Stannius corpuscle is an encapsulated organ consisting of several lobules separated by strands of connective tissue. Each lobule contains groups of columnar cells circularly arranged around a central lumen. The large round basophilic nuclei, which are basally located, exhibit marked heterochromaticity (i.e., chromatin appears in the form of strands). Portions of cells abutting the lumen are agranular and periodic acid Schiff (PAS) negative whereas basal portions are intensely colored when stained with PAS. No cytomorphological differences were noted between acid exposed and control trout corpuscles following staining with H and E or Gomori's trichrome stains. However, cursory examination of these tissues following staining with PAS indicated greater staining in acute acid exposed trout as compared to controls. As a consequence, PAS affinity was selected to assess possible effects of acute and subacute acid exposure on secretory function of the Stannius corpuscle.

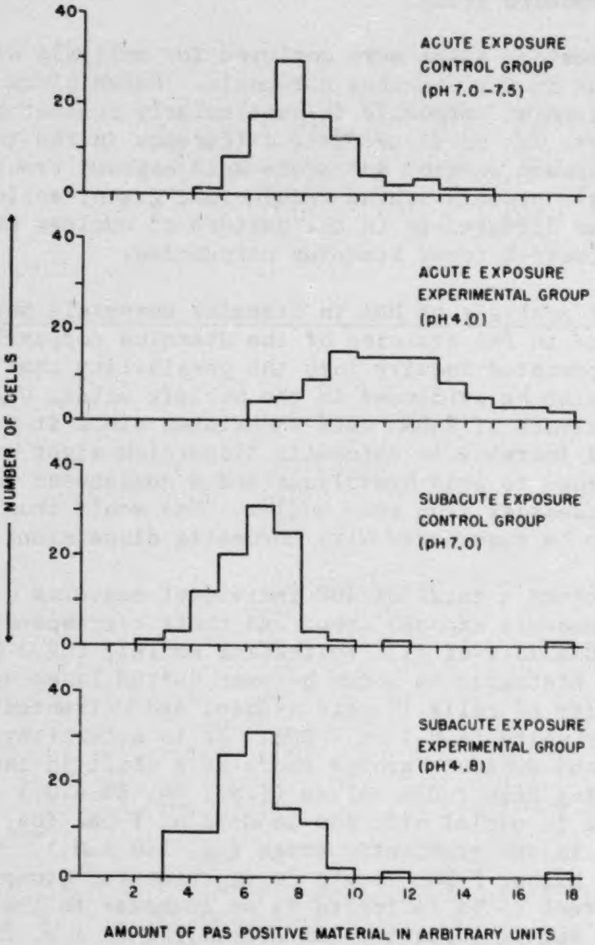

Figure 2. Periodic acid Schiff (PAS) distribution patterns for Stannius corpuscle cells of brook trout exposed to acid water for acute (4-5 day) and subacute (28 day) periods.

Cytophotometric analyses of PAS staining in Stannius corpuscles. Although PAS is a nonspecific stain (i.e., mucopolysaccharides, glycogen, glycolipids and some other compound lipids are PAS-positive), it can be used to measure relative changes in secretory material on an individual cell basis. Although PAS staining is not stoichiometric, one can assign relative numerical values to cells exhibiting decreased or increased staining affinity. As with the F-DNA data, results were expressed in the form of histogram profiles to facilitate comparison of the various experimental groups. From data summarized in Figure 2, it is apparent that Stannius corpuscle cells from trout exposed to acute acid exhibited a pronounced increase in the number of cells containing more PAS-positive material. On the other hand, no increase in relative numbers of cells exhibiting high affinity for PAS was evidenced in Stannius corpuscles of the subacute exposure group.

Several histochemical tests were employed for analysis of lipids and nuclear proteins in the Stannius corpuscle. Sudan black B showed that lipid in the Stannius corpuscle is particularly concentrated in cell membranes. There was no discernible difference in the intensity of sudanophilia between control and acute acid exposed trout. As indicated previously, basic protein stains (eosin-fast green, eosin Y, and fast green) showed no differences in the pattern of nuclear staining in acid exposed and untreated trout Stannius corpuscles.

Cytophotometric analysis of DNA in Stannius corpuscle nuclei. The pronounced increase in PAS staining of the Stannius corpuscle in acute acid exposed trout prompted inquiry into the possibility that histochemical changes might also be evidenced in the nucleic acids, DNA and RNA. Cytophotometric analyses of F-DNA were undertaken since it was reasoned that an acid induced increase in chromatin dispersion might eventuate in a lowered resistance to acid hydrolysis and a consequent increase in the net yield of aldehydes from some nuclei. One would thus expect higher F-DNA values to be associated with chromatin dispersion.

Figure 3 represents a total of 400 individual measures of nuclear F-DNA in acute and subacute exposed trout and their corresponding controls. As with the F-DNA data of gill epithelial nuclei, the 2 C or diploid segment of the histogram is shown between dotted lines and indicates that the majority of cells in acid exposed and untreated groups contain 50-80 arbitrary units (A.U.) of F-DNA. It is noteworthy, however, that in both acute and subacute groups there is a shift in the number of nuclei exhibiting high F-DNA values (i.e., ca. 80 A.U.) and a proportional decrease in nuclei with low amounts of F-DNA (ca. 40 A.U.). Very few nuclei are in the premitotic stage (ca. 140 A.U.). The number of nuclei showing higher F-DNA levels in experimental groups proved to be sufficiently great to be reflected in an increase in the average F-DNA content of the entire population of nuclei (62-72 A.U. for the acute group and 59-73 A.U. for the subacute group).

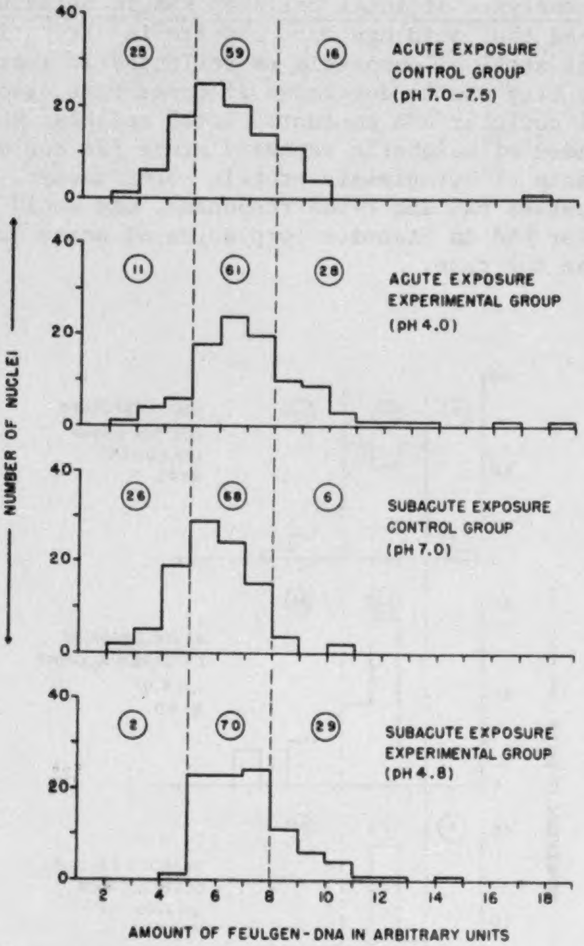

Figure 3. DNA distribution patterns for Stannius corpuscle cells of brook trout exposed to acid water for acute (4-5 day) and subacute (28 day) periods. Numbers in circled inserts refer to percentage of cells between and on each side of dotted division lines.

In brief, these data indicate that both acute and subacute acid exposure results in an increase in F-DNA content in a considerable number of Stannius corpuscle nuclei. The increase, measured on an individual nuclear basis proved to be of sufficient magnitude to be reflected in a greater mean F-DNA value of the nuclear cell population. Significantly, the mean F-DNA contents of the two acid exposed groups were of comparable magnitude.

Cytophotometric analyses of total cellular RNA in Stannius corpuscles. Having established that acid exposure results in alteration in the physical chemical state of chromatin as reflected in increased F-DNA values, the next step was to determine if these were associated with changes in total cellular RNA content. Total cellular RNA is a useful and sensitive index of metabolic activity since RNA content generally parallels synthesis of cytoplasmic protein constituents. Thus, on the basis of the observed PAS and F-DNA responses, one would expect highest levels of cellular RNA in Stannius corpuscles of acute exposed trout. This proved to be the case.

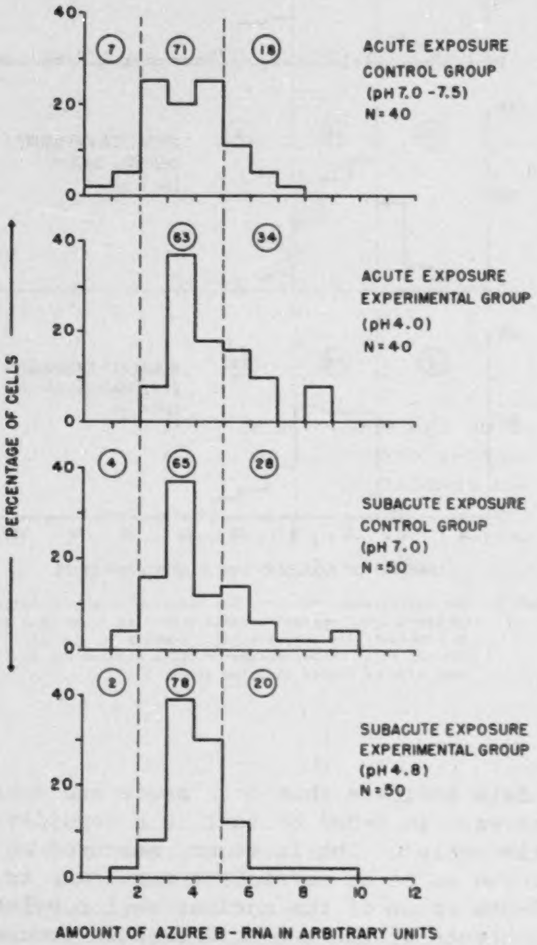

Figure 4. Total cellular RNA distribution patterns for Stannius corpuscle cells of brook trout exposed to acid water for acute (4-5 day) and subacute (28 day) periods. Numbers in circled inserts refer to percentage of cells between and on each side of dotted division lines.

Data summarized in Figure 4 represent a total of 180 measures made on individual Stannius corpuscle cells from acute and subacute acid exposed trout and corresponding control groups. The data are presented as percentages of cells containing different amounts of RNA to facilitate comparison with the F-DNA histogram data. As is evident from these data, the average content of RNA is significantly greater (45 ± 2 A.U.) in Stannius corpuscles of acute acid exposed trout than that of controls (38 ± 2 A.U.). On the other hand, subacute exposure to acid did not affect the RNA content of Stannius corpuscle cells. In fact, the average content of the subacute exposed group (41 ± 2 A.U.) was not significantly different from either of the two control groups. Therefore, the F-DNA increase noted in subacute exposure was not paralleled by an increase in cellular RNA content.

Supplemental biochemical analyses. Several biochemical measures were run to determine whether the observed histochemical changes were sufficiently great to be amenable to chemical analysis and to obtain some clues regarding the general type of secretory constituents present in the Stannius corpuscle. In one experiment, lipids were extracted from fresh, unfixed aliquots of Stannius corpuscles and the extract plated on thin layer chromatographic plates. It was determined that approximately 20 mg of tissue from 2-6 fish (number of fish dependent on their size) would yield 3-4 mg of lipid which was sufficient for thin layer chromatographic analysis of neutral and polar lipids. Acute acid exposed fish showed an increase in phospholipid content. This correlated well with the finding that the corpuscles from acid exposed fish contained a greater amount of PAS positive material since phospholipids are PAS positive. Acid exposure had no effect on the number and, presumably, the types of lipids present in the Stannius corpuscle as gauged by comparison of their mobility with known standards.

Electrophoretic analysis of gill histones was also undertaken to determine if different histone fractions were separable in gill tissue and to possibly gain insight into the nature of gill mucification. Histones were extracted from acute, subacute and control fish gills. Six histone fractions were identified by comparison with known standards (Worthington Biochemical Corp.). There were no visually discernible differences between acute, subacute, or control animals, in either number of distribution of bands. This supports the histochemical findings which indicated a lack of histone histopathology in response to either acute or subacute exposure to acid water.

Discussion and Summary. This report is concerned in part with the feasibility of using histopathological and histochemical changes to detect sublethal effects of acidity on selected tissues of fish. Histopathology, defined as the microscopic appearance of aberrant or diseased tissue, remains the classical form of diagnosing disease states, metabolic disorders and toxicant effects in vertebrate species. It also often provides one with insight into possible mechanisms involved in the induction of various types of disorders. More recently, quantitative histochemical methods have been successfully used to detect signs of incipient damage, long before cytopathological changes are microscopically evident. For example, changes in DNA, RNA, and histones, coupled with other histochemical techniques have proven especially useful in analyses of tissue dysfunction in many animal species (Wied, 1966; Wied and Bahr, 1970).

Because of the chemical and structural similarity of the basic tissue types (hemopoietic, connective, epithelial, muscle, nerve) in fish and mammals, it was reasoned that selected histochemical methods, limited largely in the past to the study of mammalian tissues, should work equally well for analyses of fish tissue. Preliminary work, together with the present study, showed this to be the case. The Feulgen reaction for DNA, azure B staining for RNA, mucopolysaccharide stains such as colloidal iron, alcian blue, and the periodic acid Schiff reaction give identical responses in fish and mammalian tissues. In fact, greater staining differences are often evidenced between organs and tissue types in an individual animal (fish or mammal) than one finds when comparisons are made of staining affinities of specified organs between animal species. This is not too surprising since the specific function of specialized cell types is often closely related to its chemical make-up. It is noteworthy that although the application of histochemistry and experimental histopathology in mammals is well established, very little is known about the histochemistry of fish tissues. In view of this, it is felt that one of the important contributions of the present study is the demonstration that a number of histochemical methods developed primarily for use with mammalian tissue can be applied to the analysis of fish tissues. Furthermore, since two of the staining reactions (i.e., Feulgen, azure B) have a known chemical basis and are thus amenable to quantitation, these can serve as excellent indices of cytopathology such as might be induced by acid exposure.

Gills were selected as one of the tissues to be analyzed because preliminary work (Plonka and Neff, 1969) established that histopathological changes in gill epithelial cells are elicited by short exposure of trout to severe acid water (pH 3.9). For example, histological examination of gill sections from acid treated trout revealed increased mucin production, disruption of lamellar integrity, "clubbing" of lamellae and the occlusion of interlamellar spaces by a coagulum of cellular debris. On the basis of microscopic study, however, it was not possible to ascertain the mechanism of pathological change, i.e., the source of the cellular debris nor the extent to which remaining supporting epithelial cells were affected by acid treatment.

The Stannius corpuscle was selected for histochemical analysis since some recent work in our laboratory revealed that shifts in electrolyte and water balance appear to be associated with acid exposure (Packer and Dunson, 1970; Vaala and Mitchell, 1970). Since Hanke and Chester-Jones (1966) and Chester-Jones *et al*. (1966) have shown that one of the major functions of the Stannius corpuscle is ion regulation, it was felt that acid induced metabolic or secretory changes in epithelial cells comprising this endocrine organ should be manifested in their histochemical staining reactions.

Although the scope of this paper is restricted to analysis of these two tissues, the work described actually forms an integral portion of a broader project concerned with "screening" histochemical responses of tissues representing most of the vital organ systems in the trout. The underlying rationale for this coordinated approach which involved the participation of a number of graduate students working on different tissues was to check for the possible existence of a "toxic response syndrome," adopting the terminology proposed by Warner (1966). There is no doubt that the physiological significance of histochemical responses can best be interpreted in light of alterations in other body systems. Thus, one of the goals of the study was to determine whether histochemical changes in the gill and Stannius corpuscle are indications of histopathology or signs of acclimation to acid exposure.

Table 6. Summary table of major findings

	Type of acid exposure			
	Acute (4-5 day)		Subacute (28 day)	
	Moderate acidity[a]	High acidity[b]	Moderate acidity[a]	High acidity[b]
A. Gross responses				
1. surface mucus secretion (macroscopic observation)	⬆	⬆⬆	↑	⬆
2. hyperventilation, loss of equilibrium, surfacing	⬆	⬆⬆	-	⬆
3. loss of coloration	⬆	⬆	⬆	⬆⬆
B. Gill changes (histological, histochemical and biochemical)				
1. increase in number of mucous cells	⬆	⬆⬆	-	⬆
2. presence of interlamellar debris	↑	⬆⬆	-	⬆
3. mucopolysaccharide staining of gill cells and interlamellar area (colloidal iron, alcian blue, mucicarmine)	↑	⬆⬆	-	⬆
4. F-DNA of epithelial nuclei	-	⬆	-	-
5. basic nuclear proteins of epithelial cells (eosin-fast green, alkaline fast green, eosin Y staining)	-	-	-	-
6. electrophoretic analyses of histones	-	-	-	-
C. Stannius corpuscle changes				
1. PAS staining	⬆⬆	⬆⬆	-	-
2. F-DNA of Stannius corpuscle cells	-	⬆	-	⬆
3. neutral lipids	-	-	-	-
4. polar lipids	-	⬆	-	-
5. RNA of Stannius corpuscle cells	-	⬆	-	-
6. basic nuclear protein (eosin-fast green, eosin Y, alkaline fast green)	-	-	-	-

[a]Moderate = exposure pH 5.0-6.5 — - no change — ↑ slight increase

[b]Severe = exposure pH 4.0-5.0 — ⬆ moderate increase — ⬆⬆ marked increase

A synopsis of the overall pattern of cytological and histochemical changes as a function of the severity of acidity and exposure duration is presented in Table 6 where relative intensities of changes are indicated by direction and width of arrows. Two conclusions are supported by these data: First, it is evident that acute exposure to moderate and high levels of acidity results in increase in mucopolysaccharide content of gill cells and increased PAS staining of Stannius corpuscle cells. Second, in gill cells the intensity of the histochemical response is much greater in trout exposed to high levels of acidity under acute conditions than in moderate acid exposed groups. In contrast, PAS staining of the Stannius corpuscle was equally intense in both moderate and high acid exposed trout. No indication was found of a progressive decline in the functional capacity of these organs following prolonged exposure to moderate and high severities of acidified water as measured by differences in RNA and histone levels.

The above findings indicate that one of the major aims formulated at the onset of this investigation was accomplished, i.e. demonstrating that histochemical tests have potential value as physiological or toxicant bioassays. Of equal importance, such tests convincingly show that acidity, per se, has no direct toxic action on internal vital organs since all of the observed changes occur as a result of respiratory and circulatory impairments. The observation that F-DNA histogram profiles of supporting cells in trout gills are identical in control, acute, and subacute acid exposed animals signifies that exposure to sublethal levels of acidity does not damage gill supporting cells. Therefore, one can also conclude that interlamellar debris seen in acute acid exposed fish probably originates from sloughed mucous cells rather than supporting epithelial cells.

On the other hand, F-DNA profiles of Stannius corpuscle nuclei of acute acid exposed trout revealed the presence of considerably more nuclei with higher than average amounts of F-DNA when compared to controls; so much so, that the average F-DNA level was significantly higher in acute acid exposed nuclear populations relative to the control nuclei. Stannius corpuscle cells of acute acid exposed fish also showed an increase in azure B-RNA when compared to controls. Actively synthesizing cells would be expected to show an increase in DNA, RNA, and cellular anabolites. The combined DNA and RNA data, therefore, indicate an increase in metabolic activity of Stannius corpuscle cells in acute acid-exposed trout. This probably represents an acute stress reaction to acid since F-DNA levels remain high in Stannius corpuscles from 28 day exposed fish whereas total cellular RNA content returned to control levels. Alterations in total cellular RNA are useful indicators of protein synthesis since all functional types of RNA are directly concerned with the mechanism whereby information stored in chromosomal DNA is made operative at the sites of protein synthesis (polyribosomes). Significantly, one finds highest levels of RNA in growing cells or when their synthetic machinery has been triggered into action by some exogenous or endogenous stimulus. Conversely, resting cells or cells in a steady state (homeostatic equilibrium) contain moderate to low levels of cytoplasmic RNA. In view of these considerations, presence of increased RNA during acute acid exposure, but not following subacute acid exposure, can be explained in one of two ways. One possibility is that fish are stressed to near their tolerance limit. A more probable explanation is that stressed fish achieve a new level of homeostasis, at least in respect to this particular gland. Data on cytoplasmic PAS content and nuclear histones support this interpretation since increased cytoplasmic content of PAS was not detected in subacute exposed fish and histone staining did not indicate a cessation or inhibition of metabolic activity.

SECTION IV

ERYTHROCYTIC INDICES OF STRESS IN ACID EXPOSED BROOK TROUT

Most workers favor the idea that fish exposed to acid pollution experience a hypoxemia and tissue anoxia due to impaired oxygen transport across the gill respiratory epithelial surface (Carpenter, 1927) since acid stressed fish often exhibit a thick mucus-like coagulum in the interlamellar spaces of gill filaments (Westfall, 1945; Plonka and Neff, 1969). However, many fish can withstand a considerable extent of gill mucification and apparently recover with no grossly detectable evidence of disability except for a marked loss in body coloration. To date, little attention has been focused on possible cumulative or long term consequences of acid-induced hypoxemia in fish subjected to sublethal levels of acidity. In homeotherms, hypoxia initiates erythropoiesis through release of an erythropoietic stimulating factor (Jacobson and Doyle, 1962). Thus, it was decided to investigate the nature of hemopoietic responses in trout subjected to near toxic levels (ca. pH 4.0-3.5) of acidity for a short term (five day) and to sublethal levels (pH 4.9) for a longer (26-28 day) duration of exposure. The specific aims of this phase of work were:

1. to characterize the peripheral hemal response during acute and subacute acid exposure by monitoring changes in selected blood parameters such as hematocrit, hemoglobin content, differential counts, and blood P_{O_2};
2. to determine if acute and subacute exposure to acid stimulates hemopoiesis as reflected in the cytochemical Feulgen-DNA profile of immature erythroid cells;
3. to investigate the influence of acute and subacute exposure to acid on the metabolism of hemopoietic cells in renal and splenic tissue as reflected in RNA content; and
4. to determine if the pattern of acid induced erythrocytic changes is the same in nonbreeding and reproductively active fish.

Methods. Two hundred and seventy hatchery-reared brook trout, *Salvelinus fontinalis*, (Research Station of the Pennsylvania Fish Commission, Pleasant Gap, Pennsylvania) 12-18 months old, weighing 130-170 g and 20-25 cm in length were used. Prior to experimentation, fish were acclimated to laboratory conditions for at least one week in 150 gallon holding tanks at 12 ± 1 C under constant aeration.

Acute toxicity studies (five days) were conducted on nonbreeding trout (spring-summer months) in a static system where water was not changed during the exposure period. Controls were maintained at pH 7.0-7.3 ± 0.1.

The procedure of acute acid exposure involved the addition every eight hours of 2-3 ml of concentrated HCl (specific gravity 1.84; 37% by weight). Within one day, pH dropped from 7.0 to 5.0 ± 0.2; on days 2, 3 and 4 water was maintained at pH 4.0 ± 0.2; and on day 5, pH dropped to 3.5 ± 0.1.

Subacute toxicity studies (26-28 days) were conducted on nonbreeding trout (spring-summer months) and reproductively active trout (fall). In addition, one study was done immediately after the breeding season (winter). These experiments were conducted using a gravity flow proportional diluter system (Mount and Brungs, 1967). Controls were maintained at pH 7.0 ± 0.1 and experimentals at pH 4.9 ± 0.1. A stock solution of 9.5 N HCl was used in this system.

Field studies were also conducted on hatchery trout placed in net traps in a stream, Upper Three Runs in Clearfield County, Pennsylvania. This stream conveniently provided water plateaus of different pH's. Controls were subjected to a pH 6.4 ± 0.2 while experimentals were exposed to pH 4.8 ± 0.2. Exposure time was approximately 24-48 hours, after which fish were transported to the laboratory for analysis.

Several peripheral blood measurements were made to determine if there were any erythrocytic adjustments associated with acute and subacute acid exposure. Blood collection was performed by severance of the caudal peduncle or cardiac puncture using heparin, ethylenediaminetetraacetic acid (EDTA), or sodium citrate as anticoagulants according to dictates of specific tests used. Blood taken by puncture of the heart ventricle was employed for hematocrit measurements using heparinized glass capillary tubes. Hemoglobin determinations were based on blood obtained by severance of the caudal peduncle using the cyanmethemoglobin method (U.S. Armed Forces Medical Journal. 1954. 5:696). Differential counts of neutrophils, lymphocytes and monocytes were made on dried blood smears. WBC/RBC and reticulocyte/RBC ratios were also calculated. Counts of immature erythroid cells per 1000 mature RBC were made on renal and splenic touch preparations stained with Giemsa (Humason, 1967). On several occasions, when enough blood was available, checks were made on the total RBC count using a standard RBC diluting pipette and Hendrick's diluting solution (Hesser, 1960).

Supplemental measures of peripheral blood characteristics included: (a) blood P_{O_2} determinations on cardiac blood using a Copenhagen radiometer oxygen electrode setup (Vaala and Mitchell, 1970); (b) blood volume measures using radioiodinated serum albumen ($RI^{131}SA$, Squibb); (c) incorporation of radioiron (Fe^{59} citrate, Squibb) into hemoglobin; (d) polyacrylamide gel electrophoresis of hemoglobin using the method of Moss and Ingram (1968); and (e) electrophoretic analysis of total histones in erythrocytes following the method of Bonner et al. (1968).

For cytochemical analyses of nucleic acids in erythropoietic tissue of trout (kidney and spleen), groups of at least six fish each were acid exposed for five and 26-28 days. Each experimental set had its own control group of fish and microscopic slides representing each animal were used for DNA and RNA analyses. Following exposure, fish were sacrificed, splenic and renal touch imprints were air dried and fixed for five minutes in absolute methanol. Individual slides from each animal were stained for morphological analysis with Giemsa (Humason, 1967), for DNA by the Feulgen procedure (Deitch, 1968), and for RNA by azure B (Flax and Himes, 1952). To minimize error, control and experimental slides for each set of experiments were stained simultaneously. Optimum hydrolysis time for Feulgen staining was determined to be 60 minutes with methanol fixed tissue when using 5N HCl at 20 C. Since azure B stains both RNA and DNA, azure B-RNA was measured on sections pretreated with electrophoretically purified, RNAase free, bovine pancreas DNAase (Worthington Corp.). In addition, one slide was always stained with Schiff's reagent. This yielded a negative Feulgen reaction, and served as a control for azure B staining. Two Feulgen-DNA controls were always run, a non-hydrolyzed control and a control in which DNA was extracted, both giving negative Schiff reactions.

A single beam Leitz microspectrophotometer was used for cytophotometric measurements of immature erythroid cells. Nuclei ranging from 4.3μ to 8.4μ in diameter represented a graded series of red cell nuclei which were individually measured for Feulgen-DNA content. The nuclear sizes were chosen to represent various erythroblast maturation stages, basophilic erythroblast to reticulocyte. Fixed numbers of each cell type were selected for measurement, to insure that histograms were not influenced by population shifts in developmental stages. Total cellular RNA (azure B) measurements were made on whole cells with nuclear diameters ranging from 4.3μ to 10.5μ which represented cells in erythroid maturation from proerythroblast to reticulocyte. Specific erythropoietic cells were identified on the basis of nuclear morphology, nuclear area and cytoplasmic tinctorial properties.

The two wavelength method of microspectrophotometry described by Patau (1952) and Ornstein (1952) was used to avoid measuring errors caused by heterogeneous distribution of stained material. Wavelengths (λ_2 and λ_1) employed for Feulgen-DNA were 550 nm and 500 nm; corresponding wavelengths for azure B-RNA were 595 nm and 515 nm.

General observations. During acute exposure (five day) of trout to near toxic levels of acidity (pH ca. 4.0), the most marked symptoms of respiratory distress were evidenced when the pH was brought down from 5.0 to 4.0 on the second day of exposure. At this time a noticeable decrease in activity was observed, the rate and depth of ventilation increased, surfacing was often noted, and fish ate poorly. Copious amounts of mucus were secreted such that a film became visible over the entire skin surface, especially in the gill area. These symptoms persisted from day two to five at which time the pH was lowered to 3.5. At a pH of 3.5 trout showed a loss of equilibrium, swimming with their ventral sides up and if left at this level of acidity died within several hours. Trout collected during the nonbreeding season and used in subacute exposure (28 day) experiments at sublethal levels of acidity (pH 4.9) exhibited the same symptoms of respiratory distress as were evidenced in acute exposure studies. Stress reactions were first noted within 24-36 hours after introduction into acidified water and persisted for 8-10 days; by the end of the second week, hyperventilation was no longer evidenced nor was there any mucus secretion present, suggesting the trout were no longer hypoxemic. Significantly, the mortality of nonbreeding trout at a pH of 4.9 was 10% during the 28 day exposure interval. In contrast, in subsequent experiments with reproductively active trout exposed to sublethal levels of acidity for 28 days, the mortality rate was extremely high, 75%. Interestingly, most of the reproductively active trout died within the first 8-10 days of exposure and showed no overt signs of acclimation to acidic conditions. No attempt was made to undertake a systematic study of behavioral response. The main intent was to confirm that moderate to high levels of acidic exposure did evoke external behavioral symptoms of respiratory distress. Tissue analyses were limited to surviving fish.

Erythrocytic responses in nonbreeding trout following acute (five day) exposure to acidity. In both short term (five day) and longer term (28 day) exposure studies, measures were usually made of three basic peripheral blood characteristics (hematocrit, hemoglobin and P_{O_2} levels) and three related hemopoietic tissue parameters (DNA content, RNA content and differential counts of erythroblast cells in renal and splenic tissue). To facilitate presentation, results in this and the succeeding subsection are presented in terms of basic and supplemental measures pertaining to (a) peripheral hemal response patterns and (b) hemopoietic tissue changes in acute and subacute exposed trout.

a. Peripheral hemal responses. Data on hematocrit, hemoglobin, blood P_{O_2} levels and peripheral red blood cell count changes in acute acid exposed trout are summarized in Figure 5 with supplemental measures of blood volume and differential white counts presented in Figure 6 and Table 7, respectively. Briefly, the major findings were as follows. After five days of exposure to near toxic levels of acidity, trout exhibited a hematocrit increase of about 17% and a hemoglobin increase of 29%. Despite these compensatory hemal changes, acid exposed trout remained hypoxemic since the P_{O_2} level was approximately 20% lower in the exposure group relative to controls.

Differential counts of Giemsa stained smears were limited to lymphocytes (which comprise about 90% of the leucocytes), monocytes and polymorphs. Basophils and eosinophils were rarely found in smear preparations of either control or experimental trout. Percentage of lymphocytes was lower whereas that of polymorphs was higher in acute acid exposed trout (Table 7). The WBC/RBC ratio was lower in acid exposed trout. Examination of Giemsa stained peripheral smears revealed an exceptionally high percentage of reticulocytes in both acid exposed (10.1 ± .5%) and control (10.0 ± .5%) trout.

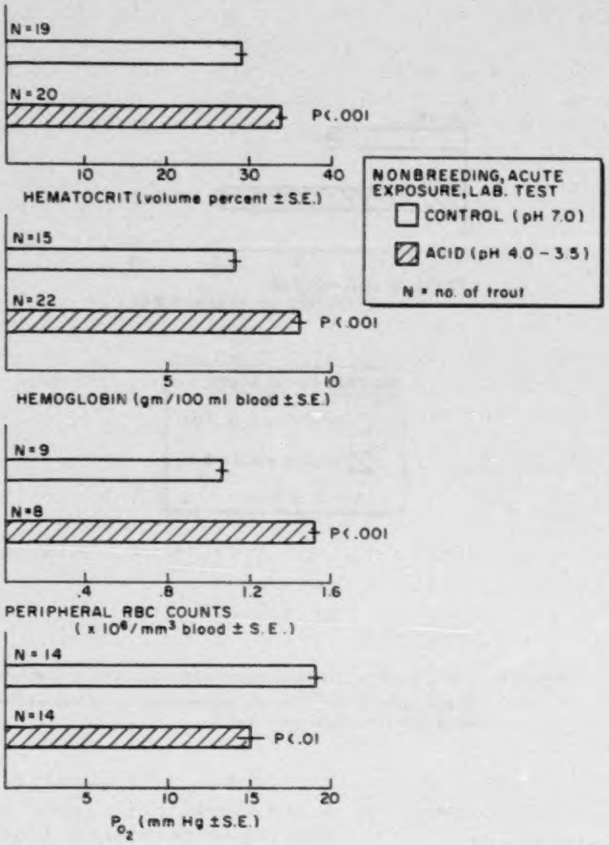

Figure 5. Hematocrit, hemoglobin, peripheral RBC count and venous blood P_{O_2} values in nonbreeding trout exposed to acid for five days (pH 4.0-3.5).

b. Hemopoietic changes. Microscopic examination of splenic and renal imprints revealed no cytomorphological differences in blast cell types between controls and acid exposed trout. Four maturation stages were readily identifiable in Giemsa stained preparations when nuclear size, chromatin dispersion and cytoplasmic basophilia were used to characterize cell types (Table 8, Figure 7). As maturation proceeds from proerythroblast to the mature erythrocyte, there is a progressive reduction in nuclear size, an increase in compaction or condensation of nuclear chromatin and increased acidophilia of the cytoplasm. The stem cell, or lymphoid hemoblast, was not characterized in this study because of controversial literature regarding its identity. It was felt that the four stages - proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, and reticulocyte - represented an identifiable

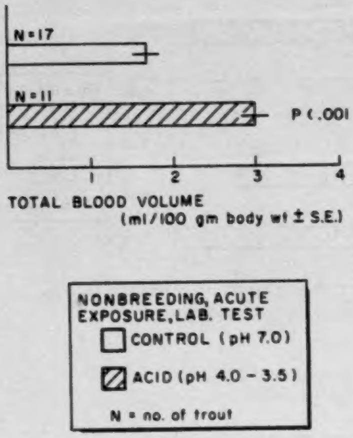

Figure 6. Total blood volume determined with radioactive iodinated serum albumin (RI^{131}SA) in nonbreeding trout exposed to acid for five days (pH 4.0-3.5).

Table 7. Differential counts in blood smears from nonbreeding brook trout exposed to acid water for five days (acute exposure) and 26-28 days (subacute exposure).[a]

Tissue	Treatment	% PMN	% Lymphocyte	% Monocyte	WBC/RBC
Blood	Acute exposure				
	control (N=10) pH - 7.0	4.1±0.7	90.0± 8.6	5.9±1.4	2.7±.4
	experimental (N=10) pH 4.0-3.5	18.6±3.7*	71.4± 6.9*	10.0±1.8†	1.9±.3†
	Subacute exposure				
	control (N=8) pH - 7.0	4.0± .6	93.1± 5.5	2.9± .3	1.4±.1
	experimental (N=8) pH 4.9	3.7± .8	93.0±11.0	3.3± .8	1.4±.1

[a] Average values (S.E.) are based on 10 differential counts per trout or approximately 100 differential counts per group. Asterisks designate significant differences at the 5% level of confidence using the t-test; † indicates p < .10, N = number of trout.

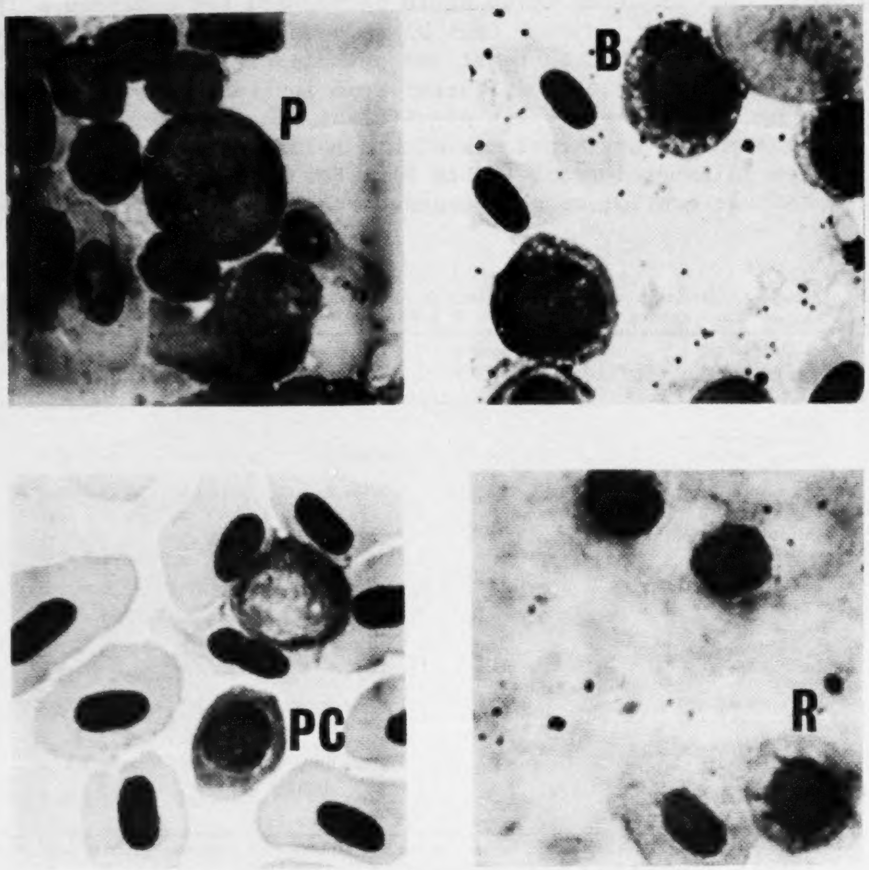

Figure 7. Photomicrographs of immature erythroid cells from a renal tissue imprint. P - proerythroblast, B - basophilic erythroblast, PC - polychromatophilic erythroblast, R - reticulocyte (Giemsa stain, 1440X).

Table 8. Maturation sequence of trout erythrocytes in renal and splenic hemopoietic tissue.

Maturation Stages	Nuclear Diameter	Chromatin	Cytoplasm
(1) proerythroblast ↓	(9.5-10.5μ)	dispersed	moderately basophilic
(2) basophilic erythroblast ↓	(7.3- 8.4μ)	moderately condensed	intensely basophilic
(3) polychromatophilic ↓	(6.0- 6.8μ)	condensed	basophilic (with faint acidophilia)
(4) reticulocyte ↓	(4.3- 5.5μ)	highly condensed	acidophilic (with faint basophilia)
(5) erythrocyte	(3x5μ)	highly condensed	acidophilic (no basophilia)

maturation sequence which could be counted and analyzed histochemically on an individual cell basis. The total number of immature cells per 1000 RBC in renal and splenic tissue from exposed trout was not significantly different from control values. However, in kidney tissue there was a significant reduction in the percentage of basophilic erythroblasts with a relative increase in reticulocytes in exposed trout (Table 9). Few mitotic figures were found in either control or acid exposed renal or splenic specimens.

Table 9. Differential red blood cell counts in renal and splenic touch preparations from nonbreeding brook trout exposed to acid water for five days and 26-28 days.[a]

Tissue and treatment groups	No. of immature RBC/1000 mature RBC	% of each type RBC precursor ± S.E.[b]			
		I	II	III	IV
Renal:					
Acute exposure					
control (N=10) pH - 7.0	170 ±13	18.8±2.3	21.7 ± 4.7	24.7 ±8.8	34.8 ±4.7
experimental (N=10) pH - 4.0-3.5	152 ±16	16.4±7.8	4.6*± 1.9	25.6 ±6.5	53.4*±6.5
Subacute exposure					
control (N=8) pH 7.0	138 ±13	12.4±3.6	17.4 ± 5.0	17.4 ±4.3	52.8 ±7.2
experimental (N=8) pH - 4.9	60*±10	10.0±1.6	21.6 ± 5.0	30.0*±5.0	38.4 ±8.3†
Splenic:					
Acute exposure					
control (N=11) pH - 7.0	180 ±15	1.7±0.5	11.7 ± 7.2	18.3 ±4.4	68.3 ±6.6
experimental (N=11) pH - 4.0-3.5	213 ±20	1.5±0.9	13.6 ± 5.1	23.4 ±6.1	61.5 ±7.0
Subacute exposure					
control (N=8) pH - 7.0	60 ± 5	6.6±1.6	33.3 ±11.6	15.1 ±5.0	45.0 ±6.6
experimental (N=8) pH - 4.9	44*± 2	11.3±4.5	9.3*± 2.2	27.2 ±6.8	52.2 ±6.8

[a] Complete count data (with standard errors) in Table 6 (Appendix). Asterisks indicate significance at the 5% level of confidence using t-test; † indicates $p < .10$; N = number of trout.

[b] Types of precursors: I - proerythroblast, II - basophilic erythroblast, III - polychromatophilic erythroblast and IV - reticulocyte.

Feulgen DNA analyses of blast cell nuclei revealed very few basophilic erythroblasts containing 4C amounts of F-DNA where C is defined as the haploid amount of DNA. When these data were plotted in the form of frequency histograms, with F-DNA levels expressed in arbitrary units (A.U.) on the abscissa and number of cells in different maturational stages on the ordinate, no differences were found between acid exposed and control trout tissue (Figure 8). Most cells contained a diploid (2C) amount of F-DNA. Only 7-12% of total blast cells in renal and splenic imprints showed interclass (3C) levels of F-DNA. It was found that late maturational stages (polychromatophilic blasts and reticulocytes) with condensed nuclei have a lower F-DNA content than the earlier basophilic erythroblast stage which contains dispersed chromatin.

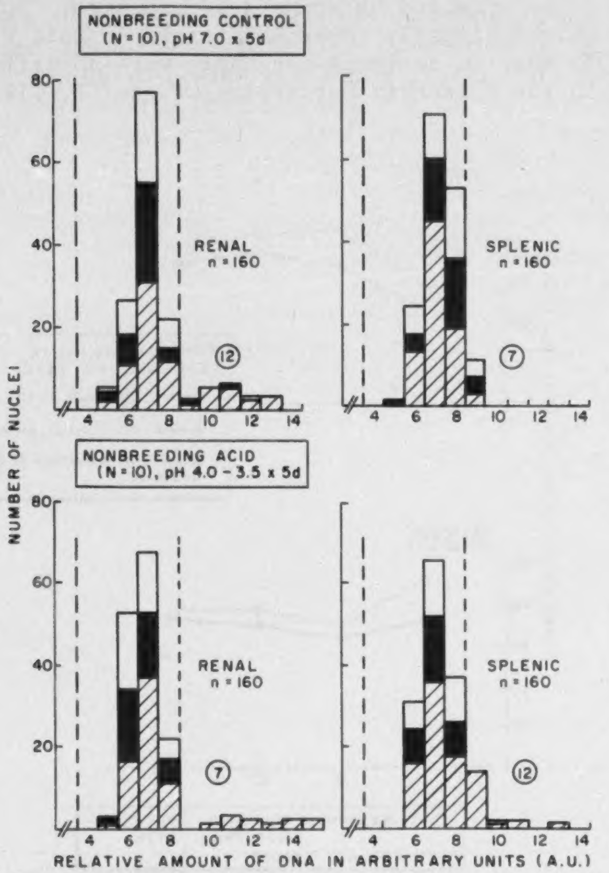

Figure 8. Feulgen-DNA distribution patterns for a mixed population of erythroid cells from renal and splenic imprints of nonbreeding trout exposed to acid for five days (pH 4.0-3.5). Dotted lines indicate the 2C category. Numbers in circles indicate percentages of cells to the right of the 2C category. Maturation stages: I(basophilic blast), II(polychromatophilic blast) and III(reticulocyte) are hatched, solid and open bar graphs, respectively.

Total cellular RNA content generally parallels the rate of synthesis of cytoplasmic protein and therefore can be used as a sensitive index of metabolic activity. For example, early stages of maturation in hemopoietic tissue of control and exposed fish often show much higher total cellular RNA levels than are found in polychromatophilic erythroblasts or reticulocytes. Cytophotometric analyses of azure B-RNA levels in cells representing stages II, III and IV of the erythroid maturation sequence revealed a 21% decrease in RNA content of basophilic erythroblasts and a 10% decrease in total cellular RNA of polychromatophilic erythroblasts in renal tissue of acid exposed trout as compared to controls (Figure 9). However, the RNA content in renal proerythroblasts and reticulocytes did not appear to be affected by acute acid exposure. Splenic proerythroblasts showed slightly lower RNA levels in acid exposed trout ($p < .05$) than in controls but there were no differences in RNA levels in the spleen in maturation stages II, III and IV.

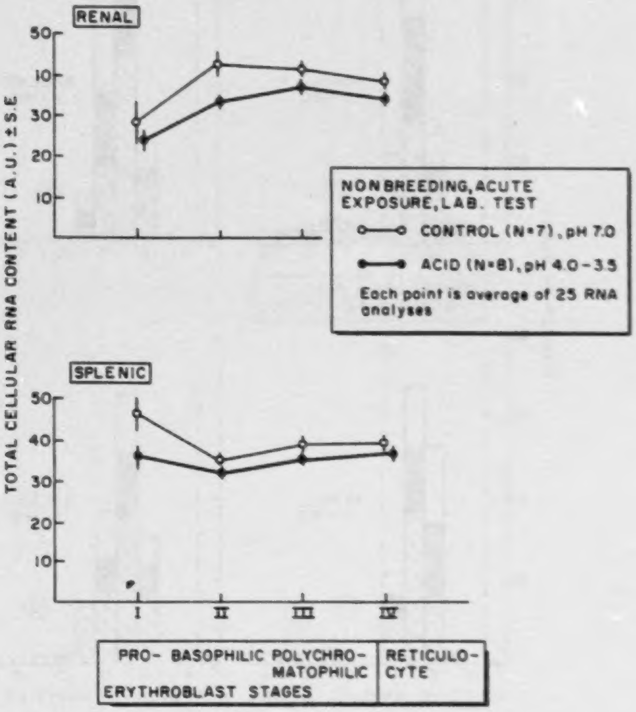

Figure 9. Total cellular RNA content (±S.E.) of erythroid maturation stages in renal and splenic imprints of nonbreeding trout exposed to acid for five days (pH 4.0-3.5).

One additional observation should be noted. Renal proerythroblasts of both control and acid exposed fish had lower RNA levels than stages II to IV. This was not evidenced in splenic proerythroblasts nor in renal or splenic tissue of controls or acid exposed trout used in subsequent longer term exposure (see Figures 9, 13, 15).

c. Supplemental measurements. Fe^{59} incorporation, which provides an estimation of the rate of iron incorporation into hemoglobin, did not differ in exposed fish from controls when both sets of values were calculated on the basis of an assumed standard blood volume. Fe^{59} uptake in control fish was 25 ± 2% and in experimentals it was 27 ± 2%. However, when actual blood volume in each group of animals was calculated, exposed trout showed an increased rate of iron uptake ($p < .001$) after five days of acute exposure (Figure 10). The same results were obtained whether radioiron uptake data were calculated on the basis of initial, final or mean body weight. Thus, for uniformity all data were recorded on the basis of final body weight.

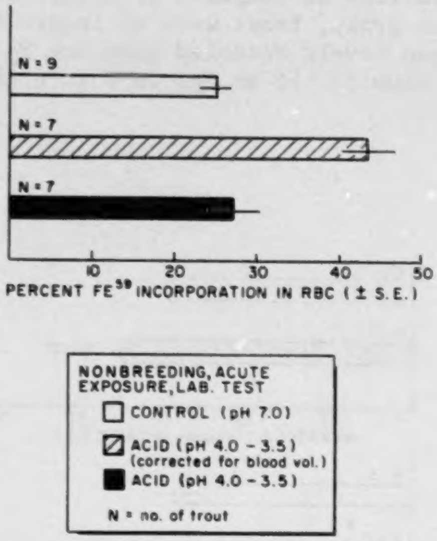

Figure 10. Radioactive iron incorporation in RBC of nonbreeding acute acid exposed trout (pH 4.0-3.5 x 5d).

Polyacrylamide electrophoretic analyses of hemoglobin in 16 blood samples from acute acid exposed and control trout revealed no difference in the pattern of electrophoretic mobility or the relative proportions of bands. Five bands were visually identified and scanned. Polyacrylamide electrophoresis of the same 16 blood samples for total histones yield two major fast moving histone fractions and two to three minor components with lower electrophoretic mobilities. Based on previous work in our laboratory using fish liver (Crissman and Therrien, 1970), three major fractions were tentatively identified as an arginine rich and two slightly lysine rich histones, with the three minor fractions representing an arginine rich and two lysine rich histones. In the acute exposure experiment, only two major fractions were resolved in both controls and experimentals and the minor arginine rich histone fraction was absent in experimental fish blood but present in controls. No attempt was made to quantify these data since pure histone preparations were not available for use as reference standards.

Erythrocytic responses in nonbreeding trout following subacute (28 days) exposure to acidity.

a. Peripheral hemal response. Measurements of hematocrit, hemoglobin and blood P_{O_2} are summarized in Figure 11. As in the five day exposed fish, hematocrit and hemoglobin levels were elevated in 28 day exposed fish as compared to controls. However, unlike the acute exposure group, trout were no longer hypoxemic. Determination of blood oxygen levels revealed that the P_{O_2} of cardiac blood samples was the same (11-12 mm Hg) in both control and 28 day exposed trout.

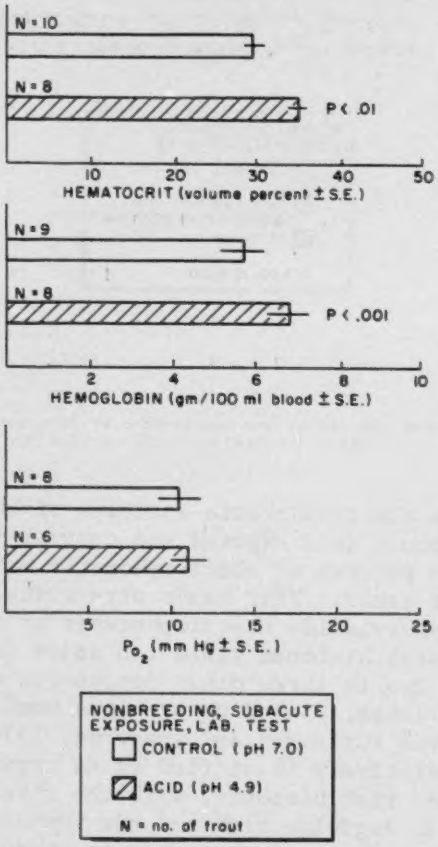

Figure 11. Hematocrit, hemoglobin and venous blood P_{O_2} values in nonbreeding trout exposed to acid (pH 4.9) for 26-28 days.

Differential counts of WBC also revealed a restoration of relative percentages of lymphocytes, polymorphs and monocytes to control levels in subacute exposed fish (Table 7). Similarly, the leucocyte to erythrocyte ratio was no longer depressed in subacute exposed trout as was the case in acute exposed fish. Reticulocyte counts (per 100 RBC) were also comparable in subacute exposed (1.7 ± .5) and control (1.1 ± .5) trout.

b. Hemopoietic changes. Differential counts of immature erythroid cells in Giemsa stained renal tissue indicated that the maintenance of an elevated hematocrit was related to a depletion of immature cells in 28 day exposed trout. There was more than a 50% reduction in the immature to erythrocyte cell ratio in subacute exposed fish. The control was 138 immature cells (stages I-IV) per 1000 erythrocytes; that for exposed trout was 60 per 1000 RBC. Percentages of maturation stages I to IV in unexposed trout renal tissue were 12, 18, 17 and 53%, respectively; corresponding values for subacute exposed trout were 10, 22, 30 and 38%. Thus, there was a relative drop in reticulocytes ($p < .10$) with a proportional increase in polychromatophilic erythroblasts ($p < .05$) in renal tissue of 28 day exposed trout.

Similar analyses of differential counts in spleen cells again revealed a lower total number of immature to mature RBC ratio in 28 day exposed trout as compared to controls. Only the percentage of maturation stage II, however, was reduced in the acid exposed group. It was further noted that the immature to mature RBC ratio in acute exposure controls in splenic tissue was three fold greater than that of controls in the 28 day exposure experiment, suggesting an atypically high level of erythropoiesis in the former group of control fish used in acute exposure studies.

Cytophotometric measurements of nuclear DNA made on blast cells in renal and splenic hematopoietic tissue of subacute exposed trout and corresponding controls (Figure 12) revealed little ploidy (1-11% of total cells measured) which was restricted to basophilic erythroblasts. There was no apparent stimulation of mitotic activity in subacute exposed fish as evidenced by lack of increased ploidy in blast cells.

Total cellular RNA was measured cytophotometrically in maturing blast cells from renal and splenic tissue imprints of subacute exposed and control fish. The proerythroblast stage in renal tissue of exposed trout as compared to controls revealed a 32% increase in total cellular RNA (Figure 13). This was not evident in proerythroblast cells from splenic tissue. However, immature erythroid cells from both hemopoietic tissue imprints showed an initially high amount of RNA which progressively decreased as blast cells differentiated into erythroblasts.

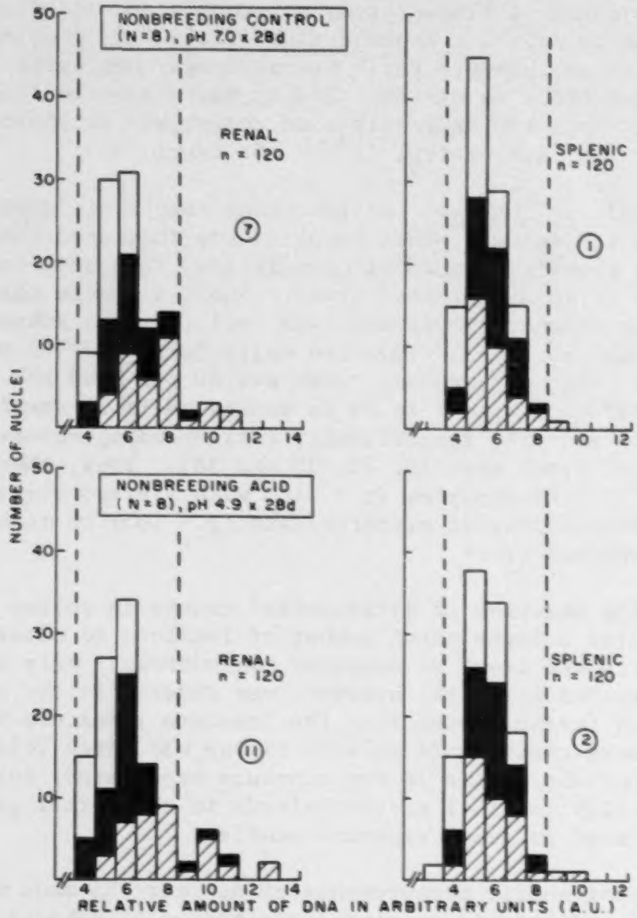

Figure 12. Feulgen-DNA analyses of erythroid nuclei in renal and splenic imprints of subacute acid exposed trout during nonbreeding season (pH 4.9 x 26-28d). Dotted lines indicate the 2C category. Numbers in circles indicate percentages of cells to the right of the 2C category. Maturation stages I, II and III are designated by hatched, solid and open bar graphs.

c. Supplemental measurements. Polyacrylamide electrophoretic analyses of blood histone and hemoglobin were again run on blood samples from eight control and eight subacute exposed trout. No differences were found in banding patterns of hemoglobin as evidenced visually and from microdensitometer scans of gels.

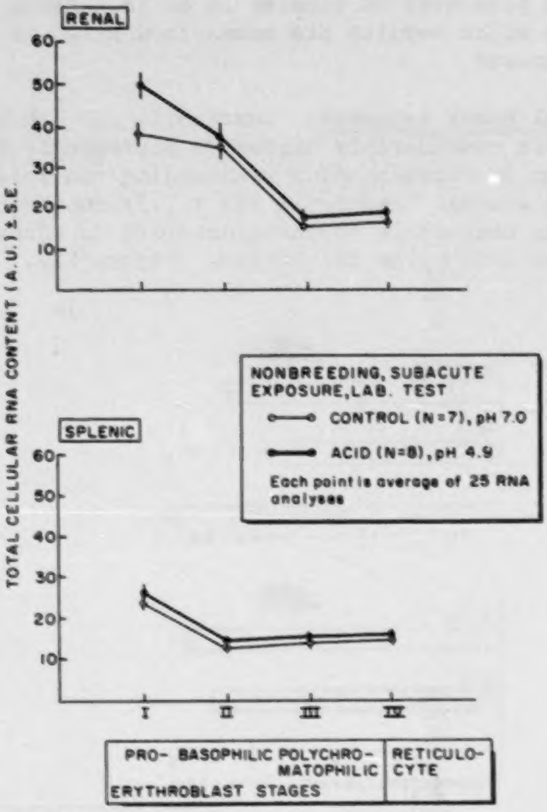

Figure 13. Total cellular RNA content (±S.E.) of erythroid maturation stages in renal and splenic imprints of subacute acid exposed trout during nonbreeding season (pH 4.9 x 26-28d).

Erythrocytic responses in reproductively active trout exposed to sublethal levels of acidity (pH 4.9) for 28 days. Since the overall pattern of acid induced hemal and hemopoietic responses observed in nonbreeding trout appeared to be related to oxygen lack, the next phase of the investigation involved the use of reproductively active trout. During the reproductively active season teleosts presumably have a much higher requirement for oxygen associated with a very high rate of metabolic activity. Thus, one might expect hemal and tissue responses to acid exposure to be more pronounced during reproductive activity. An alternate possibility would be a breakdown of compensatory hemal or hemopoietic mechanisms. Actually, the overall data indicated both the presence of exaggerated responses in selected hemopoietic parameters as well as a failure of peripheral hemal adjustments to cope with the combined stress of acid exposure and increased oxygen demands during the reproductively active period. Data on peripheral changes and hemopoietic changes which document this conclusion are presented in Figures 14 to 18. Again to facilitate presentation, the major results are summarized below in terms of selected parameters measured.

a. Peripheral hemal response. Hematocrit and hemoglobin levels were found to be considerably higher in reproductively active control trout than in corresponding nonbreeding controls (Figure 14). In fact, the average hematocrit (33 ± 1.3) and hemoglobin (7.6 ± 0.4) values are comparable to those obtained in nonbreeding trout exposed to acid water for 28 days (Figure 11).

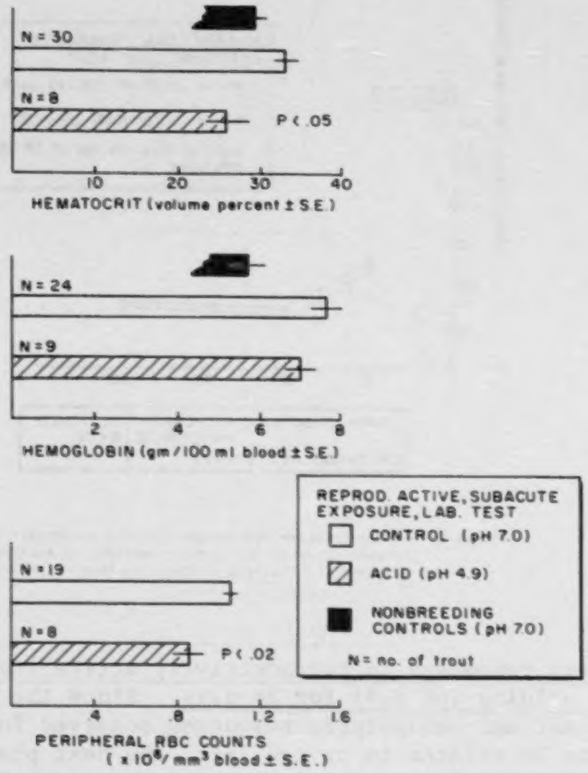

Figure 14. Hematocrit, hemoglobin and peripheral RBC count in reproductively active trout exposed to acid (pH 4.9) for 26-28 days.

Unlike nonbreeding trout, where these indices increased by acid exposure, reproductively active trout had a lower hematocrit, hemoglobin and peripheral count after 28 days of exposure. Correspondingly, the supplemental measurement of total blood volume (Figure 16) showed a drop of 11% in subacute exposed fish during reproductively active months. Differential WBC counts of blood smears revealed a higher percentage of polymorphs but lymphocyte and monocyte percentages did not change in acid exposed fish (Table 10). P_{O_2} was not measured due to the limited number of surviving trout, i.e. mortality was 75%.

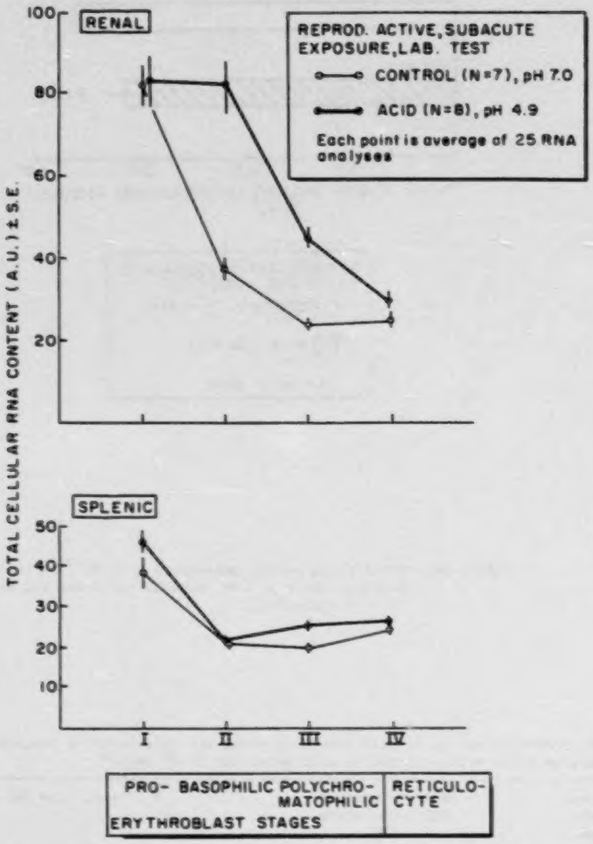

Figure 15. Total cellular RNA content (±S.E.) of erythroid maturation stages in renal and splenic imprints of reproductively active, subacute acid exposed trout (pH 4.9 x 26-28d).

Table 10. Differential counts in blood smears from reproductively active brook trout exposed to acid water for 26-28 days.[a]

Tissue	Treatment	% PMN	% Lymphocyte	% Monocyte	WBC/RBC
Blood	Subacute exposure				
	control (N=6) pH - 7.0	5.4±1.5	93.2±8.8	1.4±0.1	1.2±0.1
	experimental (N=7) pH - 4.9	10.0±1.3*	88.4±6.9	1.6±0.4	3.0±0.2*

[a] Average values (±S.E.) are based on 10 differential counts per trout or approximately 100 differential counts per group. Asterisks designate significant differences at the 5% level of confidence using the t-test. N = number of trout.

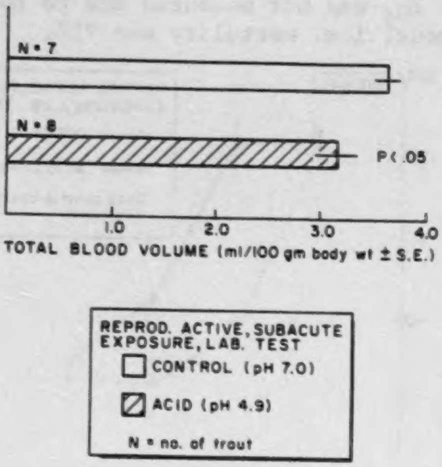

Figure 16. Total blood volume determined with RI^{131}SA in reproductively active, subacute acid exposed trout (pH 4.9 x 26-28d).

Table 11. Differential red blood cell counts in renal and splenic touch preparations from reproductively active brook trout exposed to acid water for 26-28 days.[a]

Tissue and treatment groups	No. of immature RBC/1000 mature RBC	% of each type RBC precursor ± S.E.[b]			
		I	II	III	IV
Renal:					
Subacute exposure					
control (N=6) pH 7.0	249 ±20	9.2±1.6	13.3±4.8	12.0 ±2.8	65.5 ±10.0
experimental (N=7) pH - 4.9	74*± 5	9.5±1.3	16.2±1.3	29.7*±6.7	44.6†±10.8
Splenic:					
Subacute exposure					
control (N=6) pH - 7.0	57 ± 4	14.1±3.5	12.3±5.2	17.5 ±3.5	56.1 ±10.5
experimental (N=7) pH - 4.9	49 ± 3†	22.4±4.0	10.3±6.1	20.4 ±6.1	46.9 ± 6.1

[a] Complete count data (with standard errors) in Table 7 (Appendix). Asterisks indicate significance at the 5% level of confidence using t-test; † indicates $p < .10$; N = number of trout.

[b] Types of precursors: I - proerythroblast, II - basophilic erythroblast, III - polychromatophilic erythroblast and IV - reticulocyte.

b. Hemopoietic response. Measures of immature RBC to mature RBC numbers in renal tissue indicated this ratio was much higher in breeding controls than in nonbreeding controls (see Tables 9 and 11), whereas splenic values were comparable in both control groups. This again agrees with the interpretation that during the reproductively active period there is marked augmentation of erythropoiesis. Total cellular RNA content of proerythroblasts was also greater in renal and splenic tissue imprints from reproductively active controls than in nonbreeding controls (see Figures 13 and 15).

Immature erythroid differential counts summarized in Table 11 on renal imprints from subacute exposed trout during the reproductively active months indicated a significantly lower number of immature RBC/1000 mature RBC. In splenic tissue the number of immature RBC to 1000 mature RBC also dropped in exposed trout compared to controls.

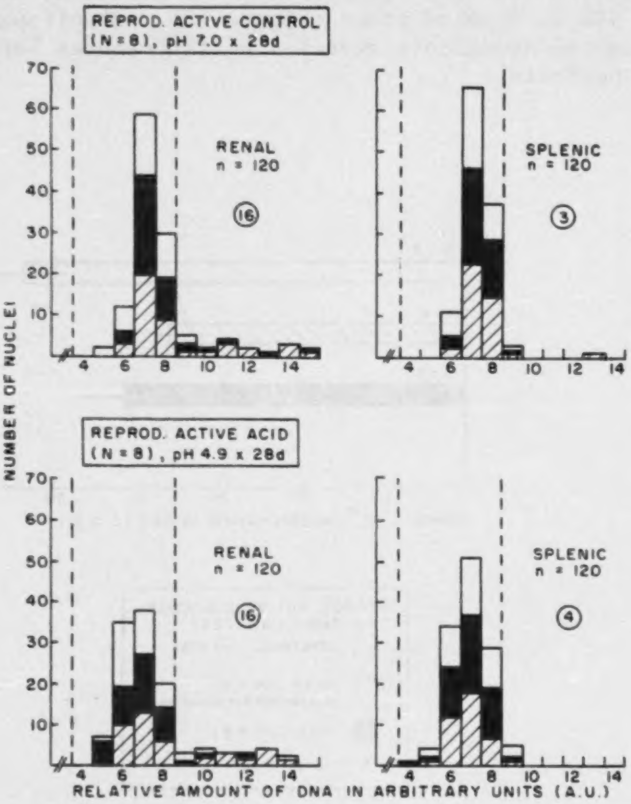

Figure 17. Feulgen-DNA measurements of erythroid blast nuclei in renal and splenic imprints of reproductively active trout exposed to acid (pH 4.9) for 26-28 days. Dotted lines indicate the 2C category. Numbers in circles indicate percentages of cells to the right of the 2C category. Maturation stages I, II and III are designated by hatched, solid and open bar graphs.

Feulgen-DNA measurements on maturing blast nuclei in renal and splenic hemopoietic tissue are summarized in Figure 17. There was no proliferative response of blast cells in reproductively active, exposed trout nor any evidence of incipient cellular damage. Azure B-RNA cytochemical measurements (Figure 15) revealed an initially high amount of total cellular RNA in proerythroblasts from renal and splenic imprints of exposed trout. RNA levels markedly declined as blast cells matured. In renal hemopoietic tissue of exposed fish, total cellular RNA showed a pronounced rise of 115% in basophilic erythroblasts and 88% in polychromatophilic erythroblasts. Even polychromatophilic erythroblasts from splenic imprints revealed a rise of 32% in total cellular RNA.

Uptake of Fe^{59} in erythrocytes of trout exposed to acid during reproductively active months dropped by 23%. When the percentage of Fe^{59} incorporation was corrected for blood volume, the decrease in uptake was 33% in exposed trout (Figure 18). Electrophoretic analyses of extracted hemoglobin revealed no differences between experimentals and controls.

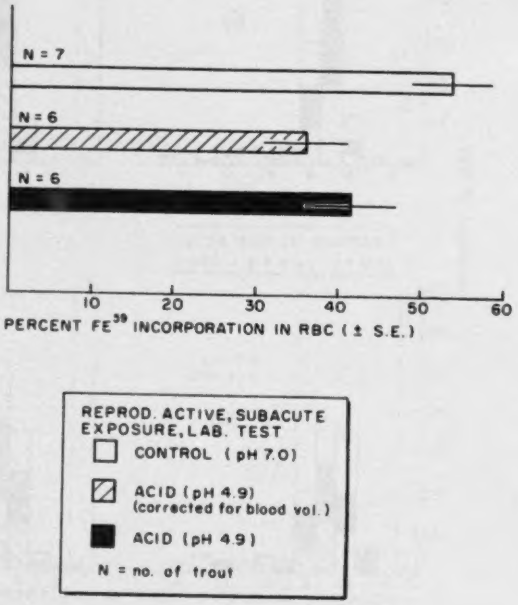

Figure 18. Radioactive iron incorporation in RBC of reproductively active, subacute acid exposed trout (pH 4.9 x 26-28d).

Hemal responses in postbreeding trout exposed to acidity for 28 days. Checks were made immediately postbreeding to ascertain the nature of peripheral hemal reactions to acid exposure when the reproductive tract was involuted, i.e. when metabolic activity associated with reproduction is again at an ebb and therefore oxygen requirements begin to approach those of the nonbreeding or reproductively quiescent trout. In brief, this group of trout presumably would be less hypoxemic than reproductively active fish during acid exposure. This proved to be the case. Mortality was again about 10% corresponding to nonbreeding trout. Hematocrit and hemoglobin content as well as the blood P_{O_2} levels approximated those of nonbreeding controls (Figure 19). Significantly, 28 day acid exposed trout in the postbreeding period showed no overt behavioral symptoms of respiratory distress and blood P_{O_2} levels were slightly higher than corresponding controls.

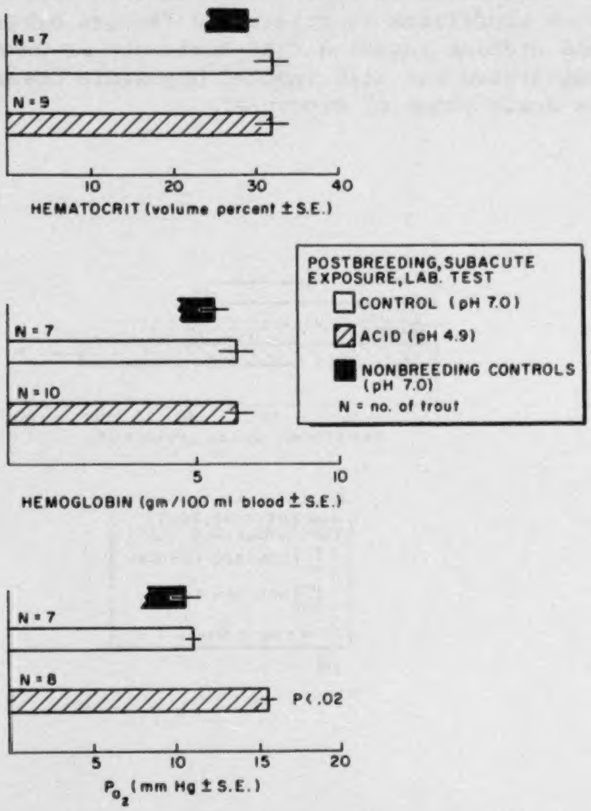

Figure 19. Hematocrit, hemoglobin and venous blood P_{O_2} values in subacute acid exposed trout immediately following breeding season (pH 4.9 x 26-28d).

Field studies of nonbreeding trout placed in acid streams. Considerable time and effort were expended in attempts to validate laboratory findings under field situations, i.e. in streams containing levels of acidity comparable to those employed in the laboratory but stemming from acid mine drainage. A number of stream sites were used and several hundred fish introduced after acclimating them to nonacid segments of an acid polluted stream. None of the acid exposed fish survived longer than one week at pH 4.8 ± 0.2. This precluded making hemal and hemopoietic measurements following 28 day intervals of exposure. Measurements of peripheral parameters following acute exposure were also limited due to the high incidence of mortality. Hematocrits from surviving exposed trout were elevated compared to controls (Figure 20). Blast cells showed no cytomorphological changes. F-DNA profiles of the immature erythroid cells revealed increased proliferation in both renal and splenic imprints from both controls and experiments (Figure 21), i.e. 40-46% of the total blast cells measured fell into interclass or 4C amounts of DNA compared to previous F-DNA profiles (see Figures 8, 12, 17), where 1-16% of the total cells measured fell to the right of the 2C category. It was felt that this stimulation in blast cells in both controls and experiments under stream conditions is related to factors other than acid exposure. Thus, field studies revealed that toxicants or unknown factors other than acidity aggravated the acid induced hypoxemia observed in the laboratory during the acute phase of exposure.

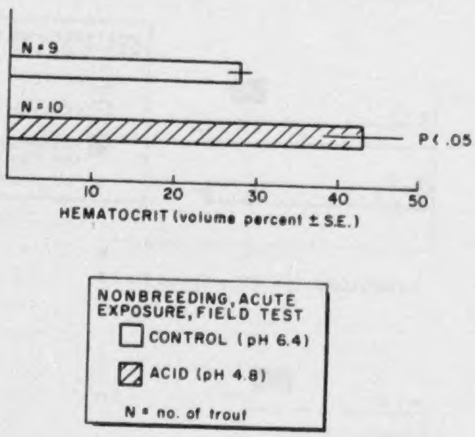

Figure 20. Hematocrit of nonbreeding trout exposed to acid (pH 4.8) for two days in the field.

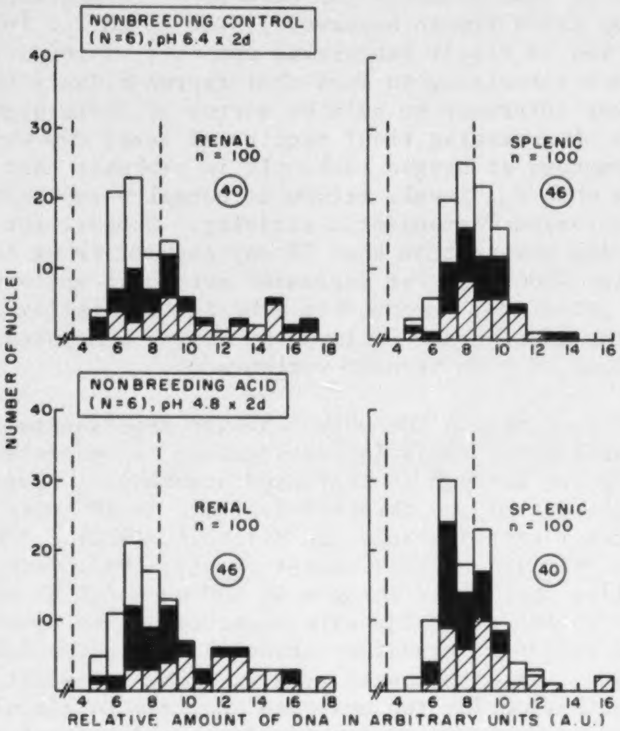

Figure 21. Feulgen- DNA distribution patterns for a mixed population of erythroid cells from renal and splenic imprints of nonbreeding, acute acid exposed trout in the field (pH 4.8 x 2d). Dotted lines indicate the 2C category. Numbers in circles indicate percentages of cells to the right of the 2C category. Maturation stages I, II and III are designated by hatched, solid and open bar graphs.

Discussion. A major contribution of this phase of work is that it elucidates certain aspects of the erythropoietic process following short and long term exposure of nonbreeding brook trout to sublethal levels of acidity. Specifically, peripheral blood measurements indicated compensatory hemal changes suggesting increased mobilization of blood stores and a hemoconcentration in both short and long term exposure. However, RNA cytochemical measurements of blast cell populations indicated that metabolic aspects of hemopoiesis were not the same in short and long term acid exposed trout. Acute exposure caused a reduction in metabolic activity (depressed RNA levels) while subacute exposure stimulated regulatory aspects of erythroid maturation. When reproductively active trout are exposed to acid, compensatory hemal responses are unable to meet higher oxygen demands stemming from increased energy expenditure (associated with reproductive changes) and decreased oxygen transport across the gill epithelium. As a consequence, there is a breakdown in peripheral adaptive blood responses, despite an exaggerated increase in RNA levels in the early blast cells of hemopoietic tissue.

It is noteworthy that although the hematocrit is elevated during acute acid exposure, trout remain hypoxemic, i.e. blood P_{O_2} levels remain sufficiently low to elicit behavioral symptoms of respiratory distress. Thus, it is not surprising to find that reproductively active trout exhibit a lower tolerance to acid by virtue of their higher oxygen requirements. Nonbreeding trout require at least two weeks before they lose overt symptoms of oxygen lack. It is probable that this coincides with the time when P_{O_2} levels return to normalcy and that this is attributable to increased hemopoietic activity. Support for this is derived from the observation that 28 day exposed trout have normal P_{O_2} levels and also show signs of increased metabolic activity in hemopoietic sites. This pattern of response to hypoxia, consisting of hemoconcentration during the acute phase followed by active hemopoiesis, is basically the same as that of warm blooded vertebrates.

Hematological findings in subacute exposure experiments indicate that the ultimate physiological basis for acclimation is an increased availability of oxygen effected through an increased hematocrit. During the onset of acid exposure, most of the observed increase in RBC mass comes from splenic and renal stores (Vaala and Mitchell, 1970). Other workers (Hall et al., 1926; Phillips, 1947; Prosser et al., 1957; Ostroumova, 1970) support the idea that rapid changes in RBC mass can be achieved in fish through mobilization of erythrocyte reserves in the spleen. However, this is probably a temporary expedient preceding the establishment of a higher level of hemopoiesis with longer term exposure to acidity. The most plausible explanation for the observed increase in circulating RBC mass in 28 day exposed trout is an increased rate of maturation and turnover of erythroid precursor cells. The basis for postulating a stepped up rate of erythroid maturation in 28 day exposed trout stems from observed changes in numbers of immature erythroid cells in kidney hemopoietic tissue. A 50% decrease was found in the ratio of blast cells to mature RBC in kidney of acid exposed fish, suggesting a faster production and output of erythrocytes into the circulation.

In keeping with the hypothesis that stimulation of regulatory aspects of cellular metabolism is associated with the erythroid maturation process in acid exposed trout, one would anticipate increased RNA levels in early blast cells. This proved to be the case. Total cellular RNA was higher in proerythroblasts in renal tissue of acid exposed trout indicating a stimulatory effect in the maturational activity of early blast cells.' Azure B-RNA profiles also revealed higher total cellular RNA in early maturation with subsequent stages showing a progressive decline in RNA. This RNA pattern, seen in both splenic and renal imprints, has been fairly well established from studies on many types of vertebrates (Cameron and Prescott, 1963; Grasso and Woodward, 1966, 1967; Torelli et al., 1964; Storti and Torelli, 1965; Iorio, 1969). In short, one aspect of acclimation is undoubtedly stimulation of the erythron, a term which sometimes is used to describe the functional hemopoietic unit, i.e. population of cells delegated with the function of RBC production. In other words, there is an apparent increase in rate of maturation of immature RBC in subacute exposed trout.

Although one would expect an increased blast cell proliferation in acid exposed trout to account for the increased hematocrit, very few mitotic figures were seen in either renal or splenic tissue. Furthermore, DNA profiles of maturation stages indicated few cells in the premitotic or mitotic state (e.g., with 3-4C amounts of F-DNA). These observations indicate that the S-G_2 phase in teleost erythroid blast cells must be extremely short and, therefore, even with a high proliferation rate very vew cells contain 4C amounts of DNA at any one time. Further investigation is needed to document this, perhaps utilizing combined techniques of autoradiography and incorporation of labelled thymidine into DNA.

Two major conclusions (based on hematocrit and hemopoietic changes) can be drawn regarding the nature of the erythrocytic response during acid exposure. Simply restated, these are as follows: (1) the acute phase of acid exposure is characterized by an increased mobilization of erythrocytic stores with no augmentation of hemopoiesis and (2) following longer term exposure there is an amelioration of hypoxemia attributable to increased hemopoiesis.

Measures of several related blood characteristics (e.g. Fe^{59} uptake, histone profiles, blast cell counts), as well as data on mortality and hemal response patterns of reproductively active and field exposed trout, provide added support to these conclusions. However, none of these supplemental measures were considered very useful in terms of providing new information or clarifying the physiological basis or pattern of erythrocyte adjustments during acid exposure. Some of the observed changes, notably in Fe^{59} uptake, in differential counts and radioactive iodinated serum albumin index (RISA) of blood volume, are difficult to interpret in the absence of information on normal levels of blood parameters being measured in teleosts.

For example, Fe^{59} incorporation (calculated on a percentage basis) was observed to be identical in short term acid exposed and control trout. This lends support to the observation that metabolic aspects of hemopoiesis and maturation of red blood cells are not enhanced but actually may be depressed during the acute phase of exposure. However, if one calculates Fe^{59} incorporation on a blood volume basis, the "apparent" rate of iron uptake, and hence rate of hemoglobin synthesis, would appear to be extremely high. The reason for this is that the use of RISA for calculating blood volume yielded erroneously high values in acute exposed trout probably because rate of dilution of the tracer compound was much higher (as a consequence of hemoconcentration) in acid exposed than in control trout. Thus, although the 24 hour interval recommended for Fe^{59} uptake (Hevesy *et al.*, 1964) and the 15 minute time span used for RISA dilution (established in preliminary studies) might be valid for trout not undergoing volume changes in blood to cell compartments, these are probably not the optimum times one should use in situations where hemoconcentration is going on. In brief, it is felt that these two tests as used have questionable validity in instances where one group is exhibiting hemoconcentration.

Differential count data of immature erythroid cells proved to be quite variable and therefore was not considered as valid as the immature to mature RBC ratio since the percentages of cells in differential counts are always relative. It is felt that asynchronous division might also cause some of the observed inconsistency in percentages of maturation stages in different groups of trout. The leucocyte differential counts on blood smears revealed a drop in lymphocytes with a rise in polymorphs in acute exposed fish with a return to normalcy in subacute exposed, nonbreeding trout suggesting that the former group of trout is more stressed. This same type of shift was observed to occur in minnows following acute acid exposure (Dolsky, 1971). A similar reaction was seen by Ostroumova (1970) in trout exposed to increased salinity. These changes are difficult to explain since the function of leucocytes in fish has not been clarified.

It was interesting to note that hematocrit and hemoglobin values from controls collected during the spawning season, a period of high metabolic activity and presumably increased oxygen demand, are comparable to hematocrit and hemoglobin content seen in nonbreeding subacute exposed trout. In addition, a considerably higher ratio of proerythroblast to reticulocyte RNA content was evident in reproductively active controls compared to nonbreeding subacute controls. This further substantiates involvement of hemopoietic tissue cells which exhibited an equally high enhancement of metabolic activity in nonbreeding long term exposed trout. The latter trout, however, could acclimate since they presumably had a lower overall tissue requirement for oxygen compared to reproductively active trout.

Exposed reproductively active trout, however, showed a failure of compensatory hemal adjustments and a high mortality. Symptoms of respiratory distress persisted throughout the exposure period suggesting trout were unable to acclimate. Peripheral measurements (hematocrit, hemoglobin content and peripheral count) all decreased. Correspondingly, blood volume was significantly below control values and radioiron incorporation was depressed. This collapse of the hemal compensatory response is suggestive of ineffective erythropoiesis. Kinetic models of erythropoiesis from Lajtha (1965) and Stohlman et al. (1962) indicate that death of a certain population of cells, i.e. cessation of mitosis and differentiation, results in ineffective erythropoiesis.

As in nonbreeding trout, DNA cytophotometric profiles of renal and splenic tissue of reproductively active trout also showed no increase in numbers of proliferating blast cells. However, a pronounced increase in total cellular RNA was evidenced in basophilic and polychromatophilic erythroblasts. This might reflect an attempt to restore the depressed erythropoietic line and suggests that the erythropoietic stimulus is operating, but some other mechanism must be malfunctioning. Significantly, even proerythroblasts in splenic (an accessory hemopoietic organ in trout) tissue imprints revealed a higher RNA content.

Studies similar to those described above were conducted under field conditions which involved the presence of water toxicants, as well as a low pH. All trout died within one week of exposure at pH levels which were not lethal under laboratory conditions (pH 4.8). Undoubtedly, additional environmental stresses somehow lessen the ability of fish to acclimate under field situations. Thus, projection of laboratory methodology to field situations must be regarded with caution. A pH of 5.0 may be tolerable under laboratory conditions in nonbreeding season, but in the field, where unknown toxicants may be present, standards must be based on more than one water quality criterion for maintenance of fish populations.

Summary. Four groups of hatchery-reared brook trout were used. One group consisted of nonbreeding trout exposed to near toxic levels of acidity for five days where pH was brought from 7.0 to 5.0 on day one, maintained at 4.0 for three days, and lowered to 3.5 on the fifth day of exposure. The second consisted of nonbreeding trout continuously exposed to sublethal levels of acidity (pH 4.9) for 26-28 days. A third group was composed of reproductively active trout exposed for 28 days at a pH of 4.9. The final group was composed of nonbreeding trout exposed to acidity under field conditions.

Hemal changes associated with acid induced hypoxemia were assessed using peripheral blood measures of hematocrit, hemoglobin content, blood P_{O_2}, and differential counts with supplementary measures of peripheral RBC counts and blood volume. The hemopoietic response pattern was characterized through Feulgen-DNA and azure B-RNA quantitative cytophotometry of developing blast cells in renal and splenic tissue imprints in addition to blast cell to mature RBC ratios in imprints. Supplemental information on hemoglobin and blood histones was obtained using polyacrylamide gel electrophoresis and determination of Fe^{59} uptake by erythrocytes.

The major results are as follows: (1) Acute exposure to near toxic levels of acidity evoked behavioral symptoms of respiratory stress and hypoxemia (low blood P_{O_2}). There was an increase in hematocrit and hemoglobin, but no evidence of increased hemopoietic activity in blast cell elements of spleen and kidney. Instead, total cellular RNA content in maturing erythroid cells in kidney was depressed in basophilic and polychromatophilic erythroblasts. (2) Following 28 days of exposure to a pH of 4.9, nonbreeding trout exhibited symptoms of respiratory distress only during the first 8-10 days of exposure and not during the last 10-28 days. After 28 days of exposure the hematocrit and hemoglobin were elevated and blood P_{O_2} levels were normal. Feulgen-DNA data again failed to reveal any increased proliferative activity; however, there was a definite increase in total cellular RNA in kidney proerythroblast cells. (3) Reproductively active trout had a very high mortality rate (75%) as compared to nonbreeding (10%) trout when subjected to a pH of 4.9. Furthermore, the surviving trout exhibited signs of respiratory distress throughout the 28 day exposure period. Peripheral blood measurements in these fish indicated failure of hemal compensatory mechanisms, i.e. the hematocrit was depressed relative to both nonbreeding and reproductively

active controls. On the other hand, basophilic and polychromatophilic erythroblasts in the kidney and spleen showed very high levels of total cellular RNA indicating a very high rate of metabolic activity in these hemopoietic sites. (4) None of the nonbreeding trout exposed to acidity under field conditions survived longer than one week in water of pH 4.8. Hematocrits were elevated in surviving exposed trout. F-DNA profiles revealed increased proliferation in both renal and splenic imprints from both control and experimental fish. (5) Results of Fe^{59} uptake, histone profiles, blast cell counts, and RISA estimates of blood volume generally provided additional support for the conclusions drawn from hematocrit and hemopoietic nucleic acid data. However, it was felt these supplemental measures did not add any appreciable information relating to physiological mechanisms involved in acid induced erythrocytic changes.

The significance of these results is that they characterize the erythrocytic response in brook trout during short and long term acidic exposure in terms of hemal and hemopoietic parameters. From this it is concluded that the most useful bioindicators of acid induced erythrocytic changes in peripheral blood are hematocrit (or hemoglobin) coupled with blood P_{O_2} measurements and related bioassays of hemopoietic tissue are azure B-RNA profiles of renal blast cells and the immature to mature RBC ratio in the kidney.

SECTION V

HEMAL CHANGES IN ACID EXPOSED FATHEAD MINNOWS AND LONGNOSE DACE

This subproject deals with the influence of acidified water on white blood cells of two species of fish, fathead minnows and longnose dace, which were exposed to sublethal levels of low pH water for acute, subacute, and chronic exposure periods. Also, histological and histochemical analyses of the spleen were performed to determine the extent of hemopoietic involvement during acid exposure. One objective of this work was to explore the possibility that splenic and hemal responses in small fish might be used as bioindicators of sublethal levels of acid contaminants in water.

No firm cytological or physiological basis is available for establishing a uniform or standardized nomenclature for specific types of leucocytes in teleost blood. As a consequence, considerable confusion exists regarding the identification of various white blood cells. In this study, the terminology for teleost blood proposed by Jackowska (1956) and Weinreb (1963) is adhered to since these workers have documented their reports with photomicrographs of the major cellular elements described.

Examination of smears of the peripheral blood of minnows and dace, treated with Wright's stain, reveals the following mature white cell types: lymphocytes, monocytes, thrombocytes, neutrophils, eosinophils and basophils. Basophils and eosinophils are very rare in the circulation of minnows, dace and other teleosts under both normal and adverse conditions. When seen, these cells are similar in appearance to their mammalian counterparts and are easily recognizable by virtue of their prominent cytoplasmic granules. Lymphocytes are the most prevalent white cells in dace and minnows as in other teleosts where the reported number is as high as 20 per 300 erythrocytes (Weinreb, 1959). They average six micra in diameter, with size and shape varying according to the amount of cytoplasm present. The large, rounded nucleus occupies most of the cell and contains distinct dark and light areas of chromatin. Larger lymphocytes tend to have more eccentric nuclei and a more granulated cytoplasm. There is no reliable cytological basis for differentiating between monocytes and large lymphocytes; hence, monocytes were not considered as a distinct cell type in the present study.

Although neutrophils are the most numerous of the granulocytes in a whole blood smear, they only number about two neutrophils per 300 erythrocytes (Weinreb, 1959). Their average size is 11 micra in diameter and they are round to oval in shape. The eccentric nucleus may be found in variable forms: round, indented, kidney shaped, or bilobed, as in higher vertebrates. The slightly acidophilic cytoplasm contains very fine acidophilic granules.

Neutrophils have been termed "scavengers of the blood stream," in teleosts as in mammals, for they exhibit great phagocytic activity and are drawn to areas of infection for this function. Neutrophils have been found in great abundance in the circulation of trout during acute inflammatory reactions (Weinreb, 1958). In view of these considerations, particular attention was focused on acid induced changes in circulating lymphocytes and neutrophils and relating these to possible alterations in Feulgen DNA and histone staining of spleen lymphocytes.

Methods. Fathead minnows (Pimephales promelas) and longnose dace (Rhinicthyes cataractae) were obtained by electro-fishing from Spring Creek, Centre County, or by net at the Pennsylvania Fish Hatchery, Benner Springs, Pennsylvania. The minnows weighed 1.9-4.8 g and varied in length from 4-7 cm. Dace weighed 8.4-11.5 g and varied in length from 7.5-10.5 cm. Tissues from fathead minnows exposed to graded severities of acidity for seven months were sent from research workers at the Duluth Water Quality Control Laboratory for processing and analysis in our laboratory.

In preliminary studies, several hundred fathead minnows and several hundred longnose dace were subjected to graded severities of pH to establish tolerance limits and mortality rates of the two fish species under controlled laboratory conditions. A continuous flow system, similar to that described by Mount and Brungs (1967) was employed to maintain a graded series of acidity in seven different tanks. Hydrochloric acid was used. The pH was continuously monitored by a multichannel recorder system. The water was constantly aerated, filtered and kept at 12 C using an automatic cooling unit. Minnows or dace were kept in mesh baskets to separate them from larger trout that were present in tanks. Fish were fed once a day in the form of dry pellets (Strike Fish Feed, Agway, Inc.). To standardize the water coming into the gravity flow diluter system it was passed through an ion exchange tank and a charcoal filter (Culligan filtration cylinders). This provided water which was free of Ca, Fe, and Cl and had a pH range of 7.5-7.8. However, since fish will not survive in the absence of any Ca, enough was added to aquaria in the form of $CaCl_2$ twice a day to maintain the Ca level at about 4-10 p.p.m. Water samples were taken from each tank and an ion analysis was performed by mass spectrometry (Appendix). Other analyses included measures of total acidity, total alkalinity, conductivity, and dissolved oxygen following procedures and recommendations outlined in "Standard Methods" published by APHA, AWWA, and WPCF. Fish behavioral changes and mortality were recorded at each pH level, and hematocrit determinations were performed on the blood of fathead minnows.

Subacute exposure studies (28 day) on a group of 49 field captured longnose dace were conducted in a gravity flow diluter system as described above. The pH's of the four experimental tanks were 5.89 ± .68, 6.33 ± .39, 6.73 ± .20, and 6.98 ± .14; the pH of the control tank was 7.1 ± .10. Blood smears were made from the fish at each pH for white blood cell differential counts. Splenic tissue was also taken from these fish for routine histological studies and cytophotometric analyses of Feulgen-DNA (F-DNA) in lymphocytes.

Two streams of known acidity (ca. pH 4.9 and 5.3) were chosen for acute exposure studies of longnose dace and fathead minnows in natural waterways: Black Moshannon Creek in Centre and Clearfield Counties; Upper Three Runs in Clearfield County (Appendix). These streams are small tributaries of the West Branch of the Susquehanna River and contain soft acid water from this sandstone area. Natural fish populations occur in the upper regions of each stream, while in the lower regions tributaries of acid mine water from strip mining operations enter the streams causing a decrease in pH levels. Control sites were established above the acid water tributaries on each stream and test sites were established below the entry of the acid mine water tributaries. Another control base used mainly for acclimation purposes was established on Spring Creek, an alkaline limestone stream (pH 7.8). About 50 fathead minnows and 20 longnose dace were placed on different occasions in each live trap at the selected sites. Traps were made of nylon net suspended from metal frames, and anchored to the bottom of the stream with rocks. Fish were temperature acclimated at the control site on Spring Creek, and pH acclimated before placement in the test areas with as little handling as possible to reduce stress. Holding pens were examined on a 6-12 hour basis. Water samples were taken from all sites and water analyses performed as described previously (Appendix). A representative number of fish was returned to the laboratory for analysis. Blood smears were taken for leucocyte differential counts, and the viscera of fathead minnows and the spleen of longnose dace were processed for histological and histochemical studies. The number of fish, exposure periods, and water conditions are found in Table 12.

Tissues from fathead minnows exposed for seven months to graded severities of acid in a gravity flow dilution system at the Duluth Water Quality Laboratory were shipped to our laboratory after being fixed in 4% formalin for 24 hours and preserved in 70% ethanol. Prior to tissue processing, while fish were cut dorsal-ventrally into four pieces; these were then dehydrated, cleared, embedded in paraffin and sectioned at six micra. Histological staining and analysis of F-DNA were performed on only those body sections containing the spleen. Blood smears of representative fish from each pH treatment group were also obtained for white blood cell differential counts.

Blood was obtained by severing the caudal peduncle of both dace and minnows and smears stained with Wright's stain. Lymphocytes, large lymphocytes (including monocytes), eosinophils, neutrophils and basophils were counted following the procedure outlined by Hessor (1960). Hematocrit measurements were performed only on fathead minnows exposed to a pH of 7.1 and 6.3 for 14 days, plus those exposed to a pH of 7.1 and 4.0-4.5 for 5.5 hours. Blood for hematocrits was taken from the caudal peduncle using heparinized capillary tubes.

Routine histological stains (hematoxylin and eosin) were used for localization of spleen and lymphocytes found in the spleen, and alternate slides were Feulgen stained for DNA. Optimum hydrolysis time was determined to be 60 minutes using 5N HCl and 20 C as outlined by Dietch (1968).

Control slides were prepared by hydrolyzing spleen sections in 5% trichloroacetic acid at 100 C for 15 minutes to remove all nucleic acid and stained simultaneously with the slides to be used for F-DNA analyses. The two wavelength method of microspectrophotometry (Ornstein, 1952; Patau, 1952) was used to calculate relative amounts of nuclear DNA on an individual cell basis. Two histochemical techniques were employed for nuclear histones: the picric acid-eosin Y method (Bloch and Hew, 1960), and the alkaline fast green method (Alfert and Geschwind, 1953). The latter stain was subsequently used for cytophotometric analysis of nuclear histone content. The reason for this was that control slides stained with eosin Y showed very bright non-specific staining after treatment with 5N HCl, 1N HCl, 0.2N HCl, and trypsinization to remove the histones. On the other hand, control slides stained with alkaline fast green showed little non-specific staining after removal of histones using the method of Swift (1964). The procedure for removing histones simply consists of placing blood smears in 0.2N HCl for two hours before fixation in 4% formalin. Formalin fixation apparently hinders HCl solubilization of histones. Again, an absorption curve for alkaline fast green was established and the two wavelength method of microspectrophotometry used for nuclear histone quantitation. For Feulgen-DNA the wavelengths chosen were 565 millimicra and 496 millimicra; for alkaline fast green the wavelengths selected were 635 millimicra and 590 millimicra.

Table 12. Stream conditions during field studies of acute acid exposed fish

	Fathead minnows			
	Upper Three Runs		Black Moshannon Creek	
	Control area	Test area	Control area	Test area
June 1-2, 1970				
pH	6.4 - 5.8	4.8	6.4 - 5.9	5.2
Temperature	48 F - 55 F	55 F	53 F - 62 F	62 F
Exposure time	136.5 hrs.	20 hrs.	137.5 hrs.	21 hrs.
No. of fish	5	5	5	5
June 3-4, 1970				
pH	6.4 - 5.9	4.9	6.4 - 6.2	5.3
Temperature	48 F - 54 F	54 F	53 F - 59 F	61 F
Exposure time	184.5 hrs.	17.5 hrs.	186.5 hrs.	13.5 hrs.
No. of fish	5	5	5	5

	Longnose dace		
	Control Area Spring Creek	Upper Three Runs Above acid	Acid Branch
pH	7.8	6.0	4.5
Temperature	64 F	60 F	60 F
Exposure time	collection site	210 hrs.	12 hrs.
No. of fish	10	10	10
Survival	10	10	0

Preliminary work. The pilot studies conducted on several hundred longnose dace and fathead minnows were designed primarily to determine approximate tolerance limits of these species to acid water, and to determine which of these two species was best suited for studies of subacute (28 day) exposures to graded severities of sublethal levels of acidity. Briefly, the major findings were as follows:

1. Water with a pH of 3.0-4.0 was extremely toxic to both species. All fish died rapidly, in less than two hours, and exhibited massive hemorrhaging at the base of pectoral fins, mouth and gill regions. This was presumed to be indicative of contact chemical injury to these vital regions. Epidermal mucification was also very pronounced over the entire body surface, including the pectoral, dorsal and tail fins. A pH range of 4.0-4.5 proved equally toxic since all fish died within the first 24 hours. Maximum survival time was 5.5 hours. Again copious skin mucification was evidenced as was massive hemorrhaging. The major behavioral response at these low pH levels (3.0-4.5) was a marked decrease in locomotor activity and an increase in opercular movement. The fish did not exhibit surfacing reactions or gasping responses observed in larger trout exposed to similar conditions.

2. At pH 5.0-5.8 mortality of the fish was still high, ca. 75%. On one occasion all minnows died at this pH, while on another occasion 57% of the dace died at this pH. However, some fish survived up to 28 days. None of the survivors exhibited contact chemical injury which resulted in hemorrhage. Mucification was extensive in all fish but transient (3-4 days) in those fish which did not die in a period of one to four weeks.

3. During the course of the above studies a number of fathead minnows obtained from the hatchery exhibited a high incidence of skin fungus and some also developed symptoms of Whirling disease or Popeye even in control tanks (pH 7.0). None of the field-captured dace exhibited these symptoms. As a consequence, dace were used for 28 day exposure studies.

4. No differences were found in the hematocrit of control and acid exposed fish. It was noted, however, that the mean hematocrit of fish captured in the fall tended to be slightly higher than that of summer-captured specimens.

5. In summary, the pilot studies established that the pH range of 5.0-5.8 is close to the tolerance limit for longnose dace, and a pH of 6.0 could be considered sublethal over a 28 day exposure interval.

Effect of 28 days exposure to graded severities of acidity on leucocyte counts and F-DNA content of spleen lymphocytes in dace. One of the most striking changes observed in the blood of acid exposed dace was a shift in the differential leucocyte count. The percentage of lymphocytes dropped from a mean of 91.3 to 52.0%, while that of neutrophils increased from 8.6 to 47.6% (Table 13). Since the responsive cell types in all experimental groups were lymphocytes and neutrophils, the standard error and level of significance are given only for these cell types. Increased acidity brought decreased in the percentage of leucocytes regardless of the time of year or the sex of the animals. Neutrophils in acid-exposed dace smears increased in number to such an extent that in one microscopic field five to six neutrophils could be counted (at a magnification of 900x), while corresponding areas of control smears normally had zero to one neutrophils.

Table 13. White blood cell differential counts of control and acid exposed longnose dace after a 28 day exposure.

Treatment	% Lymphocytes	P*	% Neutrophils	P*
pH = 7.1 No. of fish = 8	91.3 ± 9.6 s. e. = 3.6		8.6 ± 8.8 s. e. = 3.4	
pH = 7.0 ± .1 No. of fish = 7	91.7 ± 7.4 s. e. = 2.8		7.6 ± 5.2 s. e. = 2.0	
pH = 6.7 ± .2 No. of fish = 6	90.1 ± 7.6 s. e. = 2.9		9.7 ± 7.0 s. e. = 2.9	
pH = 6.3 ± .4 No. of fish = 6	94.2 ± 4.2 s. e. = 1.7		5.8 ± 3.8 s. e. = 1.6	
pH = 5.9 ± .7 No. of fish = 5	52.0 ± 11.7 s. e. = 5.2	0.001	46.6 ± 10.7 s. e. = 1.5	0.001

*Level of significance.

Data summarized in each histogram in Figure 22 represent 50 individual measurements of nuclear F-DNA in a population of splenic lymphocytes from each acid exposure group of dace. It was reasoned that the observed decrease in circulating lymphocytes in acid exposed dace might reflect a reduction in splenic lymphopoiesis or increased rate of destruction. With decreased mitotic activity one would expect to find fewer lymphocytes in the S phase of DNA synthesis (i.e. fewer cells with DNA levels exceeding 2C); increased numbers of degenerating cells on the other hand would lead to a greater number of cells with F-DNA content below the 2C level. Histograms are conventionally used to present F-DNA data on cell populations comprising specific tissues. The profile of such histograms serves as a useful index of changes in proportions of nuclei exhibiting chromatin loss (DNA values less than the 2C level) or increased mitotic activity (DNA content greater than the 2C level). To facilitate comparison of exposure groups, dotted lines were included on the histograms to designate the diploid 2C or non-replicated DNA segment of the lymphocyte nuclear population. To insure that the value assigned as the 2C level of DNA truly represented the diploid amount of DNA, F-DNA content was also measured in 50 sperm cells from these fish. As expected, the mean DNA content for the sperm cells was one half that of the somatic cells.

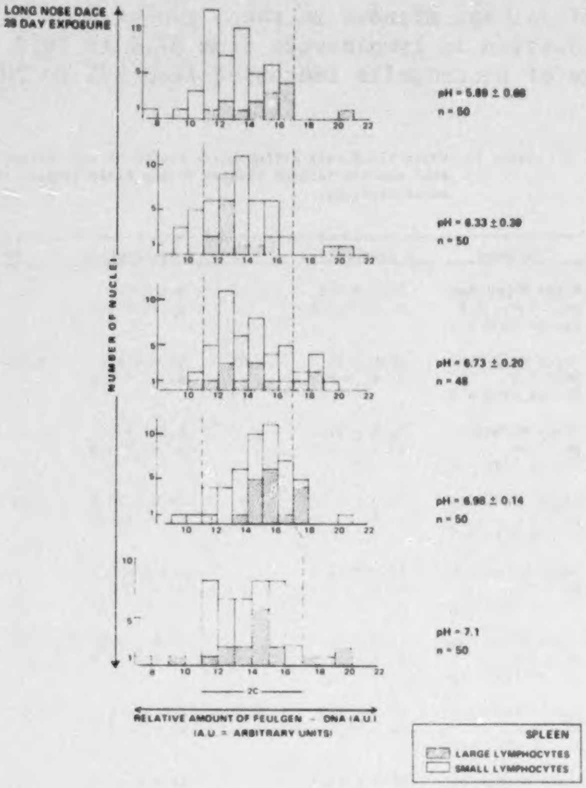

Figure 22. Histograms representing Feulgen DNA values for splenic lymphocytes of longnose dace exposed to acid water for subacute (28 day) period. Dotted lines on the histograms designate the 2C interval of F-DNA

As is evident from the data presented, there was no increase in nuclei with less than 2C amounts of DNA in splenic lymphocytes of acid exposed dace; this indicated that the rate of pyknosis was comparable in acid exposed and untreated groups. No differences were observed in the histological appearance of lymphocytes or in the extent of yellow pigment deposits in the spleen of acid exposed dace; this is also indicative of the absence of any increased lymphocytic or erythrocytic breakdown. There was also no indication that acid exposure caused any augmentation in mitotic activity. Both control and experimental spleens exhibited few if any nuclei which fell into the 4C (replicated) F-DNA category.

Field experiments on fathead minnows and longnose dace. Acute acid exposure of fathead minnows in stream traps provided as pronounced a change in the white blood cell differential as was seen in the laboratory subacute acid exposed dace. After exposure for 20 hours, fish from two acidic streams (pH 5.2 and 4.8) showed a reduction in lymphocytes from 93.4% to 58.8%, and 91.3% to 77.6%. Circulating neutrophils, on the other hand, increased from 6.6% to 41.4% and 8.3% to 24.0% (Table 14). A repeat test of fathead minnows in these two streams for 17 hours showed a percentage reduction in lymphocytes from 87.6 to 79.0 and 97.0 to 85.8. The percentage of neutrophils increased from 8.5 to 20.8 and 2.0 to 13.6 (Table 14).

Table 14. White blood cell differential counts of control and acid exposed fathead minnows during field studies of acute exposure.

Treatment	% Lymphocytes	P*	% Neutrophils	P*
Upper Three Runs pH = 6.4 - 5.8 No. of fish = 5	93.4 ± 5.8 s. e. = 2.6		6.6 ± 5.2 s. e. = 2.3	
Upper Three Runs pH = 4.8 No. of fish = 5	58.8 ± 14.3 s. e. = 6.4	0.01	41.4 ± 5.6 s. e. = 8.3	0.001
Black Moshannon pH = 6.4 No. of fish = 4	91.3 ± 11.7 s. e. = 5.9		8.2 ± 9.9 s. e. = 4.9	
Black Moshannon pH = 5.2 No. of fish = 5	77.6 ± 10.4 s. e. = 4.7	0.1	24.0 ± 10.2 s. e. = 4.6	0.05
Upper Three Runs pH = 6.4 No. of fish = 5	87.6 ± 10.3 s. e. = 4.6		8.5 ± 8.5 s. e. = 3.8	
Upper Three runs pH = 4.9 No. of fish = 5	79.0 ± 4.4 s. e. = 1.9		20.8 ± 4.0 s. e. = 1.8	0.001
Black Moshannon pH = 6.4 No. of fish = 5	97.0 ± 2.3 s. e. = 1.1	0.1	2.0 ± 1.6 s. e. = 0.7	0.1
Black Moshannon pH = 5.3 No. of fish = 5	85.8 ± 13.1 s. e. = 5.8		13.6 ± 11.3 s. e. = 5.0	

*Level of significance

Splenic lymphocyte F-DNA data from acid exposed minnows were again summarized in the form of histograms with each histogram representing 100 nuclear measurements for designated exposure groups (Figure 23). As can be seen from these data, 20 hours of acid exposure had no effect on F-DNA distribution pattern of lymphocyte nuclei, indicating no alteration of the rate of lymphopoiesis of lymphocytolysis.

Longnose dace were also subjected to acute acid exposure in selected acid streams. Figure 24 consists of three histograms each representing 100 individual measurements of nuclear F-DNA. For these measures, a concerted effort was made to select lymphocytes which contained a larger nucleus; as a consequence, the F-DNA data are representative of a nuclear population of larger, more immature lymphocytes. This explains why the shape of the histogram is not the same as that obtained for fathead minnow splenic lymphocytes. It was felt that earlier stages in lymphocyte maturation might be more sensitive to acid exposure than the more mature, smaller lymphocyte. However, as with minnows, no differences were found in the distributional pattern of F-DNA in lymphocyte nuclei of acid exposed and control dace.

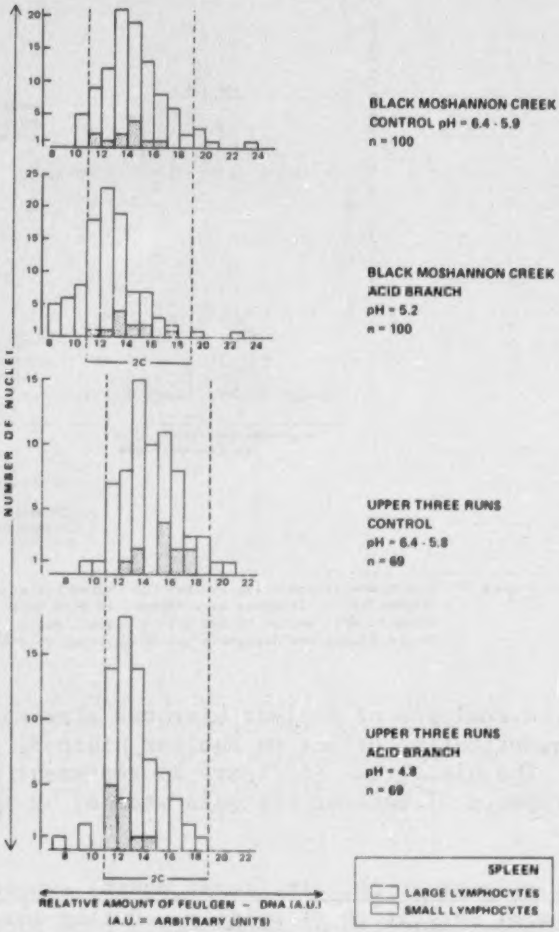

Figure 23. Histograms representing Feulgen DNA values for splenic lymphocytes of fathead minnows exposed to acid water for acute (1 day) period in two acid streams. Dotted lines on the histograms designate the 2C interval of F-DNA.

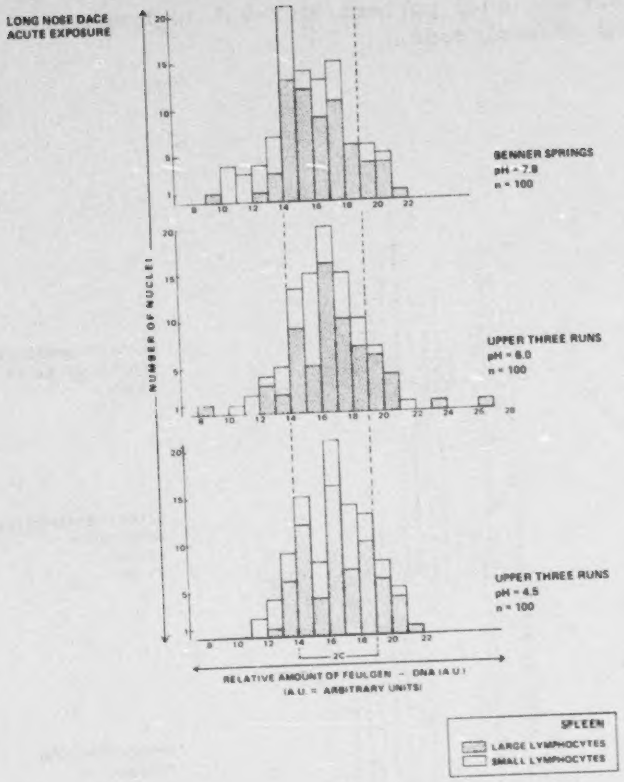

Figure 24. Histograms representing Feulgen DNA values for splenic lymphocytes of longnose dace exposed to acid water for acute (1 day) period in two acid streams. Dotted lines on the histograms designate the 2C interval of F-DNA.

Cytophotometric analyses of nuclear histones also showed that acid exposure has no quantitative effect on nuclear histones in dace splenic lymphocytes. The histograms in Figure 25 represent a total of 300 individual measurements of nuclear histones stained by the alkaline fast green method.

Hematological effects of chronic (seven month) exposure of fathead minnows to graded severities of acidity. During the course of this study, differential counts of blood smears and F-DNA analyses of splenic lymphocytes were also made on tissues from fathead minnows subjected to a seven month acid exposure to graded severities of pH (5.0 ± .1, 5.5 ± .1, 6.0 ± .1, 6.5 ± .1, 7.0 ± .1, 7.4 ± .1). Differential counts of blood smears showed that chronic exposure had no effect on the leucocyte count of minnows exposed to any degree of acidity. Blood from these fish showed a mean range of 85.8 to 91.9% lymphocytes and a mean range of 7.0 to 14.0% neutrophils over the entire pH range involved (Table 15). Blood smears stained with Wright's stain also showed no cytomorphological differences which could be attributed to acid exposure.

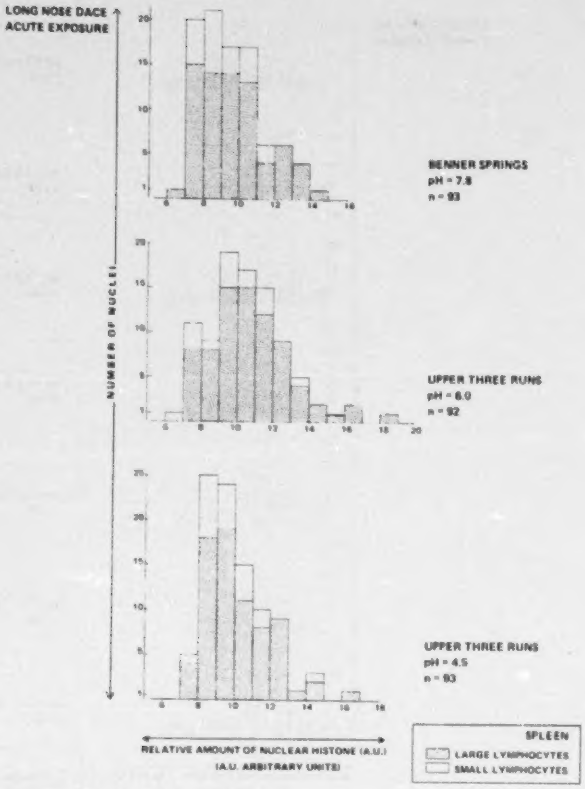

Figure 25. Histograms representing nuclear histone values for splenic lymphocytes of longnose dace exposed to acid water for acute (1 day) period in two acid streams. Dotted lines indicate the nonreplicating interval of nuclear histones.

Table 15. White blood cell differential counts of control and acid exposed fathead minnows after a 7 months exposure.

Treatment	% Lymphocytes	% Neutrophils
pH = 7.4 ± .1 No. of fish = 8	90.5 ± 5.3 s. e. = 1.9	9.5 ± 4.6 s. e. = 1.6
pH = 7.0 ± .1 No. of fish = 8	91.9 ± 8.0 s. e. = 2.8	7.9 ± 6.6 s. e. = 2.3
pH = 6.5 ± .1 No. of fish = 8	90.6 ± 6.0 s. e. = 2.1	9.3 ± 5.4 s. e. = 1.9
pH = 6.0 ± .1 No. of fish = 8	85.8 ± 9.0 s. e. = 3.2	14.5 ± 8.5 s. e. = 3.0
pH = 5.5 ± .1 No. of fish = 8	90.0 ± 7.9 s. e. = 2.8	10.0 ± 7.4 s. e. = 2.6
pH = 5.0 ± .1 No. of fish = 8	90.3 ± 4.9 s. e. = 1.7	9.9 ± 4.5 s. e. = 1.6

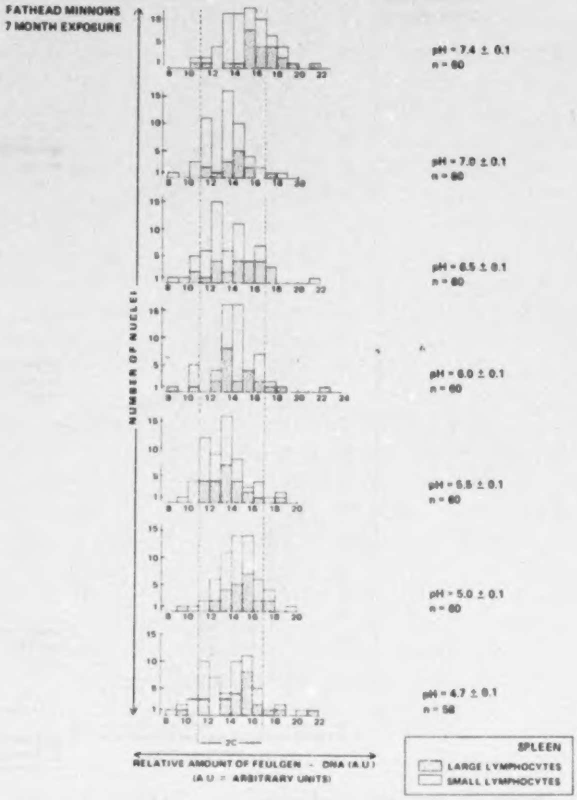

Figure 26. Histograms representing Feulgen DNA values for splenic lymphocytes of fathead minnows exposed to a graded severity of pH for a chronic (7 months) period. Dotted lines designate the 2C interval of F-DNA.

Results of F-DNA studies are presented in Figure 26. Each histogram represents 60 measurements of nuclear F-DNA from lymphocytes of designated exposure groups. Again, as in acute and subacute exposure studies, no changes were noted in the distributional pattern of F-DNA staining in splenic lymphocytes of acid exposed fish. In short, F-DNA studies indicate that neither one day nor seven months acid exposure has an effect on the physical chemical state of the chromatin of lymphocytes as reflected in their Feulgen staining affinity.

Discussion. One conclusion supported by the study is that short term exposure of minnows and dace to sublethal levels of acidity evokes circulating leucocytic changes. The functional significance of lymphopenia and neutrophilia observed in acute (ca. 24 hours) and subacute (28 day) exposed fish is difficult to explain since the role of leucocytes in fish has not been clarified. A shift in the lymphocyte:neutrophil ratio is often witnessed at the time of spawning, and in cases of parasitism and infection. Since control fish did not exhibit a lymphopenia, one can conclude that the leucocyte changes are a consequence of acid exposure. Although we have no information regarding the physiological basis for the observed leucocytic responses, it is quite likely that a disturbance in acid-base balance and electrolyte pattern is involved in triggering hemal changes. Among the factors which would tend to promote acidosis and an ion imbalance in acid exposed fish are hyperventilation, disruption or injury to the gill epithelium (Plonka, 1971) and renal changes. Packer and Dunson (1970) found that trout exposed to waters of pH less than five showed a decrease in blood pH from 7.39 to 6.97. According to A. V. Vasil'ef (1948) when the pH of mammalian blood changes, shifts in leucocyte counts are observed, with a decrease in lymphocytes occurring during acidosis. Ostroumova (1970) reported similar shifts in leucocytes in trout exposed to waters of increasing salinity which, he felt, caused a disturbance in the blood pH.

Minnows exposed to low pH for seven months did not show such leucocyte shifts. It can be assumed that these fish have acclimated. Thus, it appears that in fish, as in mammals, the early or acute stages of a toxic response syndrome are followed by a recovery phase which is characterized by a homeostatic readjustment of physiological parameters and an associated abatement or return to "normalcy" of many tissue characteristics. In related work with acid exposed trout, it was shown that histochemical changes in the gill and Stannius corpuscle, which are evoked during acute exposure, disappear after one month exposure (see Section III). Significantly, even behavioral symptoms of respiratory distress and skin mucification are transient responses, indicating fish can become acclimated to acid levels which are close to the toxic limit for a given species. Such studies emphasize the importance of using short term tissue responses as bioindicators of water toxicants rather than using tissue indices based on relatively long exposure intervals.

A few workers have studied changes in blood characteristics of fish exposed to sublethal levels of metal ion toxicants (Fujiya, 1964; Schiffman and Fromm, 1959). McKim et al. (1970) found that many physiological blood changes of trout exposed to copper were transient in nature, disappearing after extended exposure. Acid exposed trout (Vaala, 1971) often exhibit initial changes in peripheral blood parameters which disappear after a subacute exposure (see Section IV). The present study shows that for acid exposed minnows and dace, changes in white blood cell differential counts are a useful index of acidity if employed in acute exposure situations, but not in subacute (28 day) or chronic exposure (seven months).

Another important aspect of this work was the study of the role of the spleen with regard to changes in leucocyte counts of peripheral blood. The spleen is the major site of white blood cell production in the fish and varies in importance as a hemopoetic site from species to species. It was thought that acid induced lymphopenia and neutrophilia might be secondary consequences of an alteration in splenic hemopoiesis. Histological and histochemical studies showed that was not the case. Spleen tissue of acid exposed fish appeared the same as that of controls when examined microscopically following routine hematoxylin-eosin staining. For example, there was no cytological evidence of pyknosis or degeneration in lymphocytes or any indication of increased mitotic activity in neutrophilic elements.

In this study, F-DNA and nuclear histone analyses failed to show an increased rate of lymphocytolysis in the spleen of acid exposed dace and minnows after 17 hours or seven months, indicating that the potential for hemopoiesis is not impaired by exposure of fish to low pH water. Fujiya (1961) exposed Sparus macrocephalus to pulp mill waste for 24 hours and also found no evidence of histopathology in the spleen.

It is quite possible that during acid exposure there is an increased production of leucocytes in both fathead minnows and dace. Consistent with this interpretation is our finding that the number of leucocytes per 1000 erythrocytes increases from 9.0 ± 1 in control field specimens to 30.0 ± 2 under experimental field conditions. However, differential cell counts also demonstrated that there was a decrease in the percentage of lymphocytes concurrent with an increase in the number of neutrophils. Since differential counts are based on percentages of specific cell types per 100 leucocytes counted, a reduction in splenic output of lymphocytes which normally comprise about 80-90% of the circulating leucocytes would result in a relative increase in neutrophils. Weinreb et al. (1969) has recently postulated that an increase in the number of neutrophils may be due to cellular transformations or differentiations occurring in the peripheral blood. This is considered unlikely since it has not been established that leucocytes can differentiate from one stem line to another either in the peripheral circulation of mammals or poikilothermic vertebrates.

Previous work in this laboratory (Plonka, 1971; Vaala, 1971) and elsewhere (Westfall, 1945) has shown that one of the major physiological consequences of acid exposure is impaired respiratory function and a hypoxemia (Westfall, 1945; Plonka, 1971; Vaala, 1971). It has also been shown that in some species this is followed by an increased hematocrit, which possibly stems from a combination of factors such as hemoconcentration, increased erythropoiesis and mobilization of erythrocytes from hemopoietic stores. Increased mobilization of blood stores from the spleen has been cited by Ostromouva (1970) and Vaala (1971) to account for the increased red blood cell volume and increased hematocrits of stressed trout.

In conclusion, it should be stated that the lymphopenia and neutrophilia witnessed in acid exposed fish may very well be nonspecific stress responses of the fish since similar responses are known to occur in relation to breeding and parasitism. Results of a recently completed study (Vaala, 1971) indicate that trout exposed to low pH for an acute period show a lymphopenia and neutrophilia, whereas a 28 day acid exposure shows return of the leucocyte count to normal (see Section IV). In support of this are the results of our study of chronic acid exposed fish which exhibited a readjustment of percentages of leucocytes to the normal level after a seven month exposure. Therefore, this leucocyte shift must be used in conjunction with other parameters, such as histochemical changes in the gill and Stannius corpuscles of acid exposed fish, in order to establish indices of acid water pollution.

Summary. The major findings of this subproject can be summarized as follows:

1. Under both laboratory and field conditions the pH tolerance limit for both minnows and dace is approximately pH 6. Maximum survival time at a pH less than 5.0 is 5.5 hours. Workers at the Duluth Water Quality Laboratory provided us with several groups of fathead minnows that reportedly survived for seven months at pH's 4.7, 5.0, and 5.5. Blood and spleens from these fish were analyzed and constituted our "chronic" exposure group.

2. Both species exhibit behavioral symptoms of stress and contact chemical injury after acute exposure to high levels of acidity (pH 4.0, 5.0). These symptoms include decreased locomotor activity, increased secretory activity of mucous cells, and massive hemorrhaging at the base of fins, mouth and gills.

3. Subacute exposure of dace to sublethal levels of acidity (pH 5.8 to 7.0) and acute exposure of fathead minnows to acidity under field conditions evokes a shift in the leucocyte differential counts resulting in a lymphopenia and a neutrophilia.

4. However, chronic exposure of fathead minnows to graded levels of pH (7.4, 7.0, 6.5, 6.0, 5.5, 5.0, 4.7) showed no relationship between the differential count and severity of acidity. Percentages of lymphocytes and neutrophils are identical in both control and acid exposed groups.

5. No histopathological changes are found in the spleen of dace or fathead minnows following acute, subacute or chronic exposure to acidified water. Histochemical analyses of both Feulgen-DNA and nuclear histone content of splenic lymphocyte also indicate an absence of any increase in mitotic or degenerative changes in these hemopoietic cells.

6. Changes in leucocyte counts of the peripheral blood is a useful short term index of acid contamination in water.

SECTION VI

HEPATIC INDICES OF STRESS IN ACID EXPOSED BROOK TROUT

Previous studies showed that acute exposure of brook trout to low pH water evokes a toxic response syndrome which involves behavioral symptoms of respiratory distress, increased secretory activity of gill mucous and Stannius corpuscle epithelial cells (Section III), decreased efficiency of respiratory gas exchange and hypoxemia (Section IV). However, with prolonged exposure, fish exhibit adaptive responses as evidenced by increased hemopoietic activity, normal pO_2 levels and normal behavioral activity (Section IV). It was felt that acclimation of fish to oxygen lack must necessitate compensatory adjustments in tissue metabolism. In this respect, the liver, as a key organ in metabolic regulation should reveal, at the cellular level, alterations in DNA, RNA and histone staining which might be used to assess the metabolic state of the fish during long term exposure to low pH water.

Many studies attest to the involvement of DNA in the regulation of cell and tissue metabolism. Recent studies by Garcia (1967, 1969a, 1969b) and others (Noeske, 1971) have shown that small but measurable differences in nuclear Feulgen-DNA (F-DNA) staining occur in cells exhibiting different degrees of chromatin dispersion, with dispersed chromatin exhibiting slightly higher F-DNA contents than compact chromatin. Furthermore, biochemical studies by Littau *et al*. (1964) have shown that dispersed chromatin provides a more efficient template for RNA synthesis than compact chromatin. One can postulate therefore that more dye binding sites are available in dispersed chromatin of metabolically active cells; such cells should exhibit a greater Feulgen staining affinity than metabolically inactive cells. Another study (Bloch, 1966) indicates that changes in histone composition can also be correlated with functional changes in cells.

The purpose of this project is to determine whether acute (3-5d) and subacute (28d) exposure of brook trout to acid water results in detectable changes in Feulgen-DNA (F-DNA), Azure B - RNA, fast green-histones (FG-histones) or histone fractions in liver parenchymal cells and to assess the utility of such changes in detecting metabolic impairment of liver tissue. One reason for undertaking this work was to evaluate the potential of using liver histochemical changes as rapid and sensitive bioassay methods for acid contaminated water.

Methods: Two hundred and fifty seven brook trout, *Salvelinus fontinalis*, (Research Station of the Pennsylvania Fish Commission, Pleasant Gap, Penna.), 12-16 months in age, weighing 50-80 g and measuring 15-18 cm in length were used. Prior to experimentation, fish were acclimated to laboratory conditions for one to two weeks in 150 gallon holding tanks at 12 ± 1 C under constant aeration. Fish were fed dry pellets (Strike Fish Feed, Agway, Inc.) once daily.

Acute toxicity studies (five days) were conducted in a static system where water was not changed during the exposure period. Forty control (pH 7.0-7.3 ± 0.1) and sixty experimental fish were used. Acidity of experimental tanks was maintained at a pH of 4.0 ± 0.2 using HCl. On day 5, the pH was dropped to 3.5 ± 0.1. An additional 16 control and 16 acid exposed trout were also used in tracer studies dealing with effects of acute acid exposure on liver histones and liver lipids.

Subacute exposure studies (28-30 days) were conducted using a gravity-flow proportional diluter system (Mount and Brungs, 1967) to maintain a constant flow of graded levels of acidity into a series of five aquaria. The pH's of the five tanks (continuously monitored 6.6 ± 0.3 and 7.1 ± 0.2. Temperature was maintained at 12 C by an automatic cooling unit. Water samples were taken from each tank and ion analyses performed by mass spectrometry. Other analyses included measures of total acidity, total alkalinity, conductivity and dissolved oxygen following procedures and recommendations outlined in "Standard Methods" published by APHA, AWWA and WPCF in 1965. A total of 73 fish were used.

Several acute exposure experiments were also conducted under field conditions. Hatchery trout were placed in net traps in a stream, Upper Three Runs in Clearfield County, Pennsylvania. Natural fish populations occur only in upper regions of this stream, since in the lower region a tributary of mine acid water from a strip mining operation drains into the stream. A control site was established above the entry of the acid mine water tributary. Twenty two control fish were subjected to a pH 6.4 ± .2 while 30 experimental fish were exposed to pH 4.8 ± .2 under field conditions. Exposure time was approximately 24-48 hr, after which fish were transported to the laboratory for analysis.

Livers were excised from experimental and control fish and small portions fixed in phosphate buffered 4 per cent formaldehyde (pH 7.0) for 24 hr, dehydrated, embedded in paraffin and sectioned at 6 microns. Other portions, used in biochemical studies, were frozen and stored at -20 C.

Individual slides from each animal were stained with hematoxylin and eosin for morphological analysis (Humason, 1967), by the Feulgen procedure (Deitch, 1968) for DNA and for nuclear histones by the alkaline fast green procedure (Alfert and Geschwind, 1953). Control and experimental sections were stained simultaneously to minimize error. Optimum hydrolysis time for Feulgen staining was determined to be 45 min with formalin fixed liver using 5N HCl at 20 C. Two Feulgen-DNA control slides were always run, a nonhydrolysed control and a control in which DNA was previously extracted by hydrolysis in 5 per cent trichloroacetic acid at 95 C for 15 min. Both types of controls gave negative Schiff reactions. Histones were extracted from some tissue sections by treatment in 1 N HCl for 3 hr at 20 C. These control slides showed no alkaline fast-green staining. Spectral absorption curves of F-DNA and fast green histones in liver nuclei are shown in Figure 27.

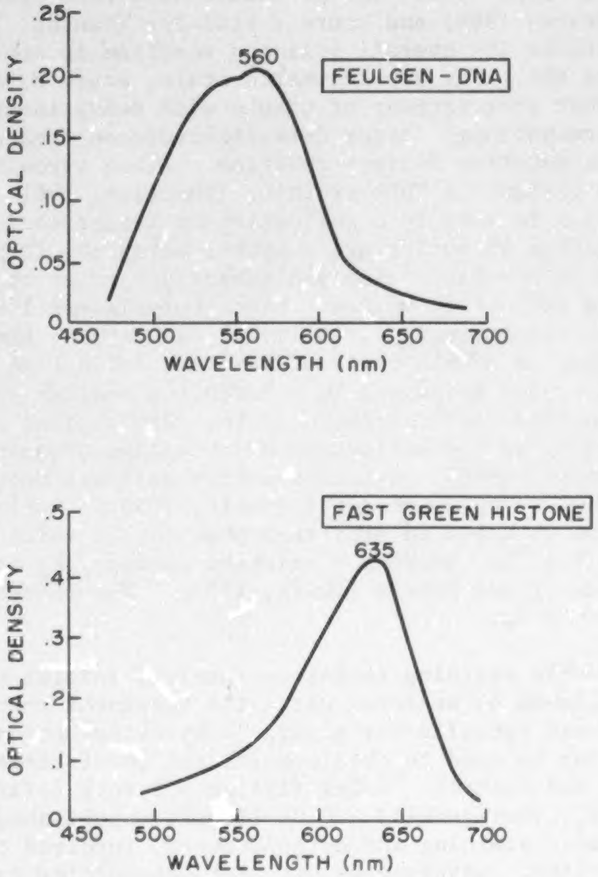

Figure 27. Spectral absorption curves of Feulgen-DNA and fast green histone complexes in trout liver nuclei.

The eosin-fast green technique was also used to differentiate arginine-rich and lysine-rich histone types on the basis of nuclear staining (Bloch, 1966). The staining reaction is semiquantitative since analysis is based on counts of the nuclei exhibiting pink, green or purple staining.

Two quantitative histochemical procedures were employed, each of which requires sequential staining of tissue with two different dyes. Using cytophotometry, quantitative comparisons of two chemical components can be made in the same cell. One procedure used FDNB (fluorodinitrobenzene) (Bloch and Brack, 1964) and azure B staining (Vaughn, 1966). Under proper conditions the overall staining reaction is specific for (lysine + tyrosine) and RNA. The metachromatic stain, azure B, stains both DNA and RNA so that pretreatment of tissue with deoxyribonuclease is required for RNA determinations. After deoxyribonculease treatment, control slides give a negative Feulgen reaction. Since tyrosine accounts for less than 10 percent of FDNB staining (Therrien, 1967), intensity of tissue staining is largely a reflection of lysine content. The relative lysine/RNA ratios in nuclei are computed using the formula E_{400}(lysine)/ E_{560}(RNA) where E = extinction and subscripts refer to wavelengths used for obtaining extinction values. Since cytoplasmic lysine and RNA also contribute to the staining intensity of each stain, the following formula is used to obtain corrected nuclear extinction values: $E_n = E_s - (1 - p/d)\ E_c$, where E_n = corrected nuclear extinction, E_s = uncorrected nuclear extinction, p is the mean optical path, d = section thickness and E_c is the extinction of cytoplasm adjacent to the nucleus (Swift and Rasch, 1956). Cytophotometric analyses were performed using the plug method of cytophotometry (Swift, 1950). Nuclear lysine or RNA content can be obtained in arbitrary photometric units (A.U.) using the formula, $M = 2/3r^2E_n$, where M = relative content, E_n = nuclear extinction and r = radius of the sphere (Swift, 1950). For spherical nuclei, the formula is $M = r^2E_n$.

The second double staining technique involves initial staining of tissue with FDNB followed by staining using the Sakaguchi reaction (Bloch, 1966). These stains are specific for (lysine + tyrosine) and arginine, respectively, and may be used to obtain molar ratios of these components in nuclei using the formula: moles (lysine + tyrosine)/arginine = 1.14 [E_{400}(FDNB)/E_{520}(Sakaguchi)] - 0.08 (Bloch, unpublished). Correction for cytoplasmic staining and cytophotometry involved the same procedures described earlier. Wavelengths used for determining extinctions were 400 nm for FDNB and 520 nm for the Sakaguchi stain. Spectral absorption curves for the two double staining procedures are shown in Figure 28.

Cytophotometric measurements of parenchymal liver nuclei were made with a single beam microspectrophotometer using the two wavelength method (Patau, 1952, Ornstein, 1952) for nuclear Feulgen-DNA and fast green histone. The standard plug method (Swift, 1950) was also employed in some cases. For Feulgen-DNA analyses, the wavelengths chosen were 560 nm and 495 nm; for fast green, the wavelengths selected were 640 nm and 675 nm.

Supplemental measurements included: (a) electrophoretic analyses of histones, (b) histone acetylation measures, (c) lipid chromatography and (d) qualitative histochemical study of lipids and glycogen. Procedures for these are briefly given below.

Liver histones were extracted as described by Bonner et al. (1958) and protein content estimated by the procedure of Lowry et al. (1951).

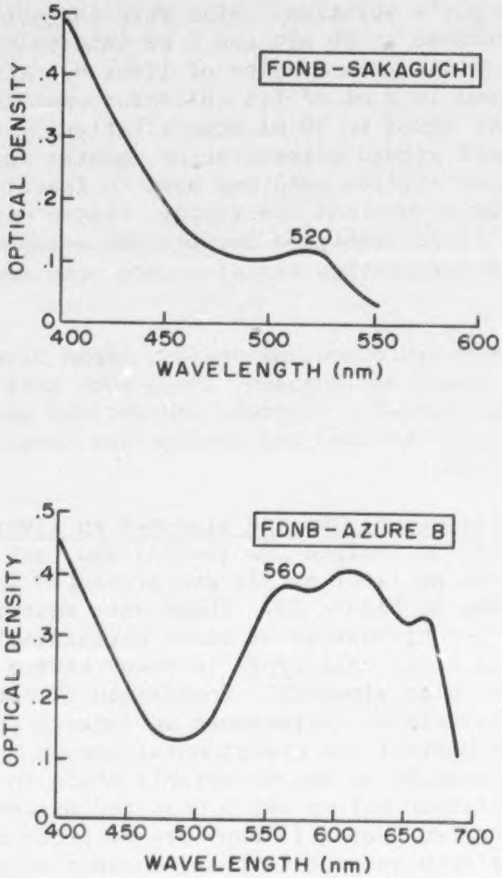

Figure 28. Spectral absorption curves of FDNB-azure B and FDNB-Sakaguchi complexes in trout liver nuclei.

Histone samples were dissolved in 10 M urea to a final concentration of 1 µg/µl. Polyacrylamide gel electrophoresis was used to identify on each gel. After electrophoresis, gels were stained for 18 hr in a 0.5 percent solution of 7 percent acetic acid. Some gels were stained with eosin-fast green (0.1 percent eosin, 0.1 percent fast green) in 4 percent formalin (pH 8.1) for 18 hr at 4 C and destained in 4 percent formalin. Microdensitometer tracings of stained gels were made using a Gilford densitometer. Gels stained with amido black were scanned at a speed of 1 cm gel/min at a wavelength of 660 nm, with a full scale deflection of 2.5 O.D. Eosin-fast green stained gels were scanned at 500 nm (eosin) and rescanned at 640 nm (fast green).

Eight control and eight experimental fish were injected intracardially with 5 µC of ^{14}C-acetate (sodium salt, specific activity 20 mc/mM) in 0.1 ml of fish Ringer's solution. Fish were subjected to acute acid exposure and sacrificed at 30 min and 1 hr intervals. Liver lipids were extracted from one gram samples of liver by method of Folch *et al.* (1957) and dissolved in 5 ml of 1:1 chloroform-methanol. Aliquots (500 µl) of each sample were added to 10 ml scintillation fluid and samples counted in a Packard liquid scintillation counter for 10 min. Thin layer chromatography (silica gel) was used to fractionate liver lipids (100 µl) into polar or neutral fractions. Plates were stained with iodine vapors and lipid fractions located and scraped into vials containing 10 ml of scintillation fluid; counts were made for 10 min intervals.

Two semiquantitative histochemical stains, Sudan Black B and the periodic acid Schiff (PAS) reaction (Humason, 1967) were used for staining lipids and glycogen, respectively. Glycogen content was assessed by visual comparison of diastase treated and nondiastase treated tissue sections following PAS staining.

Cytophotometric analyses of DNA and histones in liver nuclei of acid exposed trout. Data on Feulgen DNA (F-DNA) and fast green histone (FG-histone) content of liver nuclei are presented in the form of frequency histograms in Figure 29. These data represent 600 individual measurements (100 per histogram) of liver parenchymal nuclei. No attempt was made to analyze other cell types in liver tissue such as connective tissue cells or vascular elements. Comparison of both the DNA and histone profiles reveals no differences in average contents of F-DNA and FG-histone between control and experimental groups. Neither acute nor subacute exposure results in any detectable shift in the number of nuclei with F-DNA or FG-histone values which deviated markedly from control levels. This indicates that acid exposure of trout does not materially affect liver cells with respect to their nuclear affinity for either the Feulgen stain or the fast green stain.

The unimodal F-DNA histograms of control as well as experimental fish indicate most cells contain 2 C (unreplicated diploid) amounts of DNA. The absence of a 4 C category indicates that few nuclei in fish liver exhibit premitotic activity. The data thus reveal that neither acute nor subacute exposure stimulates mitotic activity or induces cell damage in trout liver. It is noteworthy that profiles of F-DNA and FG-histone histograms are strikingly similar indicating that in brook trout, as in other vertebrate species, the DNA and histone levels parallel one another. This suggests a close functional relationship between DNA and histones.

Cytophotometric analyses of RNA, (lysine + tyrosine), and arginine in liver nuclei of acid exposed trout. Since no detectable changes were found in total F-DNA and FG-histone, studies were designed to clarify the role of RNA and histones in liver nuclei of acid exposed trout. Lysine-rich histone fractions are known to inhibit DNA-dependent RNA synthesis. On the basis of this, it was postulated that if acid

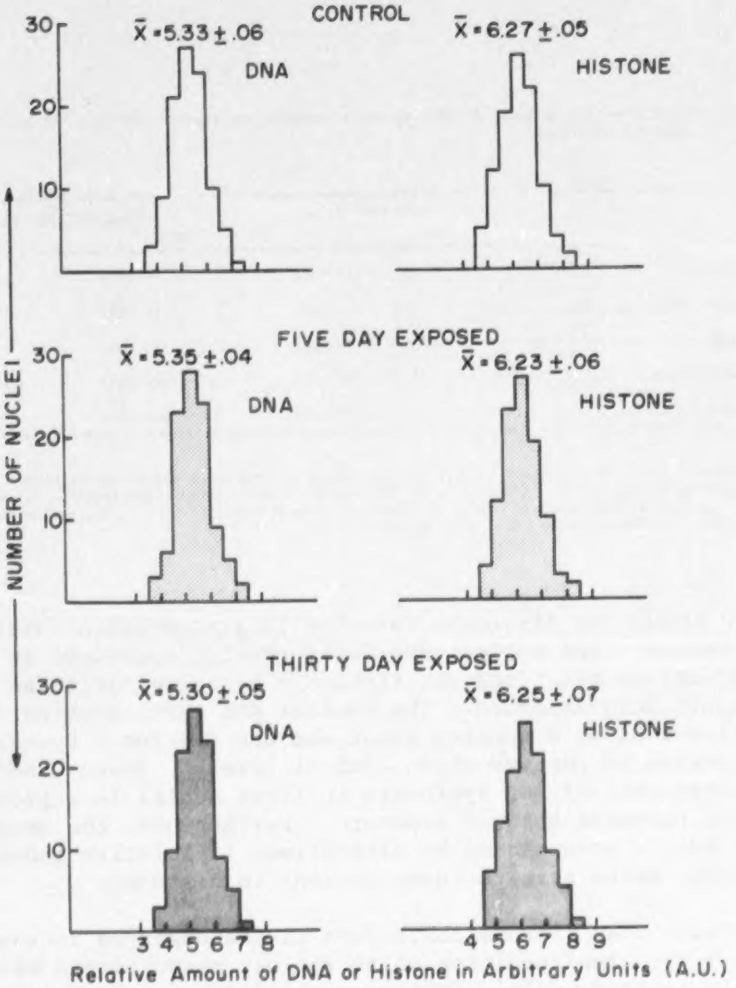

Figure 29. Frequency distribution profiles of Feulgen-DNA and fast green histone stained liver nuclei of acid exposed trout.

exposure impairs or depresses liver metabolism, this should be reflected in changes in levels of nuclear RNA, possible accompanied by changes in the two major amino acid residues of histones, lysine and arginine. Cytophotometry of liver sections stained by the FDNB-azure B and FDNB-sakaguchi reactions supported this postulate.

The RNA data and the (lysine + tyrosine)/arginine molar ratios are presented in Table 16 while the comparison of both sets of data is seen in Figure 30. It is evident that both acute and subacute exposure of trout results in a decrease in nuclear RNA with a concomitant increase in the (lysine + tyrosine)/arginine molar ratio. Nuclear RNA decreases by 43

Table 16. Cytophotometric measures of RNA, lysine + tyrosine and arginine in liver nuclei of acid exposed brook trout*

	Control Trout	Acid Exposed Trout	
		5 day	28-30 day
RNA (A.U.)	2.3 ± .12 (40)	1.3 ± .08 (40)	1.2 ± .08 (40)
(lysine + tyrosine)/RNA	1.9 ± .07 (40)	2.7 ± .11 (40)	2.9 ± .12 (40)
(lysine + tyrosine)/arginine	2.5 ± .08 (40)	5.0 ± .12 (40)	4.1 ± .11 (40)
lysine + tyrosine (A.U.)	2.3 ± .08 (40)	3.8 ± .12 (40)	3.0 ± .11 (40)
arginine (A.U.)	1.1 ± .03 (40)	0.8 ± .03 (40)	0.8 ± .03 (40)

*number of nuclei measured are in parenthesis; (lysine + tyrosine)/RNA is dimensionless being equal to the extinction at 400 nm over that at 560 nm; the (lysine + tyrosine)/arginine ratio is in moles; A.U. = arbitrary photometric units. All experimental values differ from controls at the one percent level of confidence (using Students t test).

per cent while the (lysine + tyrosine)/arginine ratio doubles after acute acid exposure. The nuclear RNA level remains depressed in liver tissue of 28 d exposed trout and the (lysine + tyrosine)/arginine ratio doubles after acute acid exposure. The nuclear RNA level remains depressed in liver tissue of 28 d exposed trout and the (lysine + tyrosine)/arginine ratio remains 64 percent above control levels. These findings indicate that curtailment of RNA synthesis in liver nuclei is a prompt and sustained response to acid exposure. Furthermore, the decrease in nuclear RNA is accompanied by alterations in relative amounts of the two major basic amino acid residues present in histones.

To determine what factors contribute to the observed increase in the (lysine + tyrosine)/arginine molar ratios, measurements were then made of double stained nuclei. This permitted direct comparison of the content of (lysine + tyrosine) with that of arginine in a given nucleus. These data also appear in Table 16 and Figure 30. It is evident that increased (lysine + tyrosine)/arginine ratios in both experimental groups are due to an increased (lysine + tyrosine) content and a concomitant decrease in arginine content of liver nuclei.

In brief, the above data indicate that acid exposure of trout results in decreased nuclear RNA levels with corresponding increased (lysine + tyrosine) content and decreased arginine content in liver nuclei. Although the (lysine + tyrosine) data do not reflect the nuclear lysine-rich histone levels _per se_, the fact that much of the nuclear lysine is a component of histone and that tyrosine accounts for less than 10 per cent of this fraction suggest that the increased (lysine + tyrosine) values are largely due to increased lysine-rich histone levels.

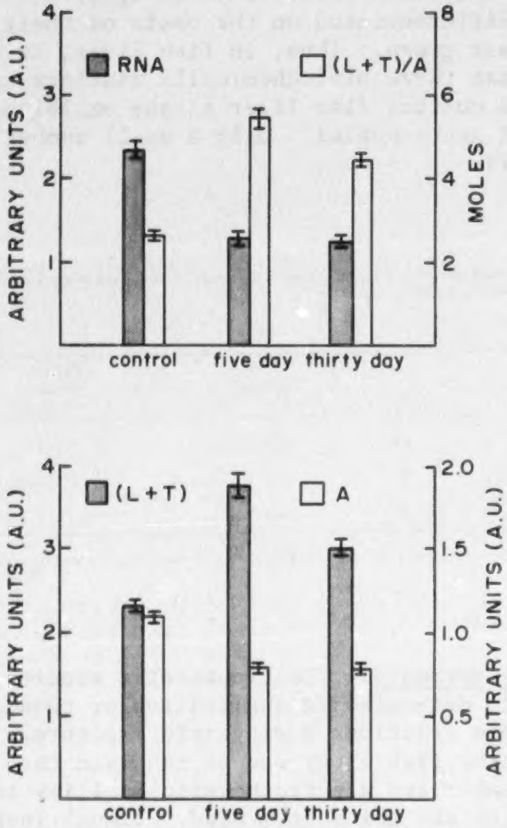

Figure 30. Effects of acid exposure on RNA, (lysine + tyrosine)/arginine ratio, lysine + tyrosine, and arginine levels in trout liver nuclei.

Cytochemical analyses of nuclear histones in liver nuclei of acid exposed trout. Having established that changes in protein lysine and arginine occur in liver nuclei during acid exposure, it was decided to employ the eosin-fast green stain which, in many instances, has proven useful for differentiating lysine rich and arginine rich nuclear histones. Nuclei containing an abundance of lysine rich histones and/or exhibiting a decrease in RNA synthesis often show a preferential affinity for eosin. Increased eosinophilia, however, was not evidenced microscopically in intact nuclei of acid exposed trout tissue despite a subsequent finding that electrophoresed histone extracts from experimental trout livers were more eosinophilic than controls.

Microscopic analysis of eosin-fast green stained liver tissue from acid exposed and control trout revealed three spectrally distinct cell types which could be differentiated on the basis of their nuclear affinities for eosin and fast green. Thus, in fish liver, as in mammalian species, there are at least three histochemically distinct cell types. Both acid exposed and control fish liver tissue exhibited an equal abundance of both pink and green nuclei. Only a small number of nuclei stained purple (Table 17).

Table 17. Relative numbers of pink, green and purple staining nuclei in acid exposed trout liver (eosin-fast green stain)

Exposure	n*	Mean % Pink Nuclei ± S.E.	Mean % Green Nuclei ± S.E.	Mean % Purple Nuclei ± S.E.
Controls	15	31.8 ± 1.6	60.7 ± 1.9	7.5 ± 0.4
Acute (5 d)	10	32.6 ± 1.7	60.2 ± 1.4	7.2 ± 0.5
Subacute (30 d)	10	31.5 ± 1.4	61.5 ± 2.0	7.0 ± 1.1

*n refers to the number of trout; at least 100 nuclei were counted per liver section.

Supplemental measurements. Electrophoretic studies of liver histones were conducted to determine if qualitative or quantitative changes occur in various histone fractions during acid exposure. It was found that the histones in control fish liver can be resolved into six major bands which correspond in number and electrophoretic mobility to calf thymus histones when examined with the gel system used. Visual inspection as well as microdensitometer tracings of the gels reveals the absence of one arginine rich band and a quantitative increase in a slower moving lysine rich band in gels of both the acute exposed and subacute exposed trout (Figure 31). It appears there is a relative increase in the lysine rich fraction at the expense of the arginine rich fraction, with no marked change in total histone. These results support cytophotometric data which indicated that acid exposure resulted in increased lysine and decreased arginine.

All six electrophoretically separated histone fractions of control trout liver stain well with either eosin or fast green when used independently; however, all fractions stain somewhat more intensely after fast green staining than after eosin staining. Eosin stained histone fractions show no absorbance at 640 nm whereas the absorbance of fast green stained histone fractions at 500 nm is negligible. For this reason, the E_{640}(fast green)/E_{500}(eosin) can be used as an index of preferential staining affinity in gels. However, gels must be stained with eosin-fast green in a formalin solution as bands diffuse from the gels in the absence of fixative. Eosin-fast green stained histone fractions exhibit colorations ranging from purple to violet depending on the content of each stain. No bands stain pure pink or green.

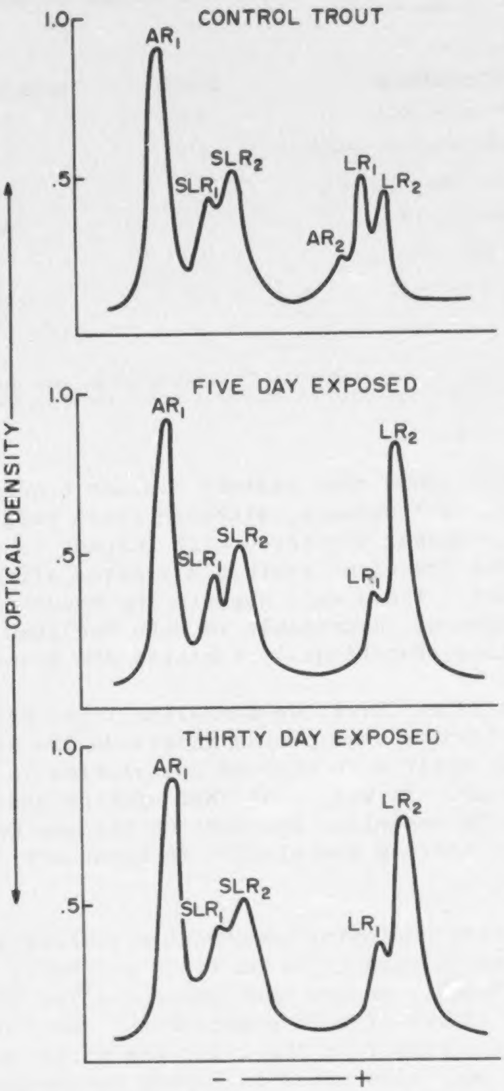

Figure 31. Microdensitometer tracings of electrophoresed histone fractions from control and acid exposed trout liver.

Histone gels of acute acid exposed and control trout were stained with eosin-fast green, scanned at 500 nm (eosin), rescanned at 640 nm (fast green) and the E_{640}/E_{500} ratios computed (Table 18). It was found that the histone bands show a greater affinity for fast green than

Table 18. Comparison of extinction ratios, E_{640}/E_{500}, of various eosin-fast green stained histone fractions from trout liver extracts.

Histone Fraction	Control	Acute acid exposed
Arginine rich_1 (AR_1)	5.4	4.6
Slightly lysine rich_1 (SLR_1)	6.3	5.4
Slightly lysine rich_2 (SLR_2)	8.9	5.2
Arginine rich_2 (AR_2)	4.2	absent
Lysine rich_1 (LR_1)	5.7	7.3
Lysine rich_2 (LR_2)	7.0	5.5

*extinctions (E) were measured at 640 nm and 500 nm for fast green and eosin, respectively; fractions obtained by polyacrilamide gel electrophoresis.

eosin; it was also found that certain histone bands are more eosinophilic than other bands. Furthermore, although three resolvable histone fractions (AR_1, SLR_1, SLR_2) appear unaltered with respect to electrophoretic ability, all three fractions exhibit a greater affinity for eosin in acid exposed trout. These data support the hypothesis that some eosinophilic component, detectable in both isolated histone fractions and tissue sections, functionally inhibits RNA transcription.

Since previous studies involving mammalian liver have shown that increased acetylation of histones accompanies increased RNA synthesis, another exploratory study dealt with histone acetylation in the liver of acid exposed brook trout. It was found that histone acetylation also occurs in fish liver as in mammalian species; no changes were noted in the extent or rate of histone acetylation in trout after acute exposure for 30 min and one hr.

An exploratory study employing radioactive acetate as a lipid precursor was also conducted to shed light on lipid synthesis in liver during acute acid exposure. Results showed that incorporation of ^{14}C-acetate into lipids occurs in livers of both experimental and control fish. A time lag in release of lipids from the liver was noted in experimental fish. Some differences were also noted in lipids synthesized and released by the liver; control lipids were predominantly free fatty acids and triglycerides while experimental lipids were mainly phospholipids and to a lesser extent free fatty acids and triglycerides.

Since both lipid content and glycogen content are often used as parameters of the functional state of liver, a number of liver tissue specimens, from control and acid exposed fish were stained with Sudan Black B for lipids and the PAS reaction for glycogen. Positive staining was obtained with each procedure. No visually detectable changes in Sudan Black B staining were evidenced after either acute or subacute exposure. A slight decrease in PAS staining was noted after acute acid exposure; however, no differences between control and experimental fish were discernible after subacute exposure.

Discussion. One contribution of this subproject is that it demonstrates that acid exposure impairs liver function in brook trout and that the site of impairment is in the nuclear machinery involved in regulation of synthetic processes. Specifically, in acid exposed trout nuclear regulatory mechanisms responsible for initiating and controlling protein synthesis are inhibited or possibly impaired. This is reflected in a decreased content of nuclear RNA which could eventuate in a decreased potential for synthesis of cytoplasmic protein constituents by liver tissue. Data obtained from electrophoretic analyses of nuclear histones further suggested that the possible agent effecting the observed decrease in nuclear RNA may be a lysine rich histone. Lysine rich histones are known to be involved in suppression of DNA-dependent RNA synthesis (Huang et al. 1964) and such histones were found to be increased in liver nuclei of acid exposed trout.

An equally important aspect of this study concerns the physiological significance of these types of liver alterations. There is no question that any inhibition of RNA synthesis at the nuclear level would probably result in a reduction in most, if not all, aspects of liver function (i.e. oxidative metabolism of nutrients, inactivation of toxins, energy stores, etc.). Under such circumstances one would anticipate a reduced tolerance of trout to other toxicants or to environmental stresses which would call for increased levels of liver metabolism.

Related findings in our laboratory support this interpretation. For example, with both brook trout and longnose dace, we observed that levels of acidity which are sublethal under laboratory conditions are toxic under field situations where acid water contains appreciable concentrations of metal ion toxicants. It was also found that levels of acidity which nonbreeding trout tolerate under laboratory conditions prove to be very toxic to reproductively active trout which presumably have much higher energy requirements (Section IV).

In another respect, however, a reduction in liver metabolism could temporarily increase tolerance of fish to hypoxemia during the acute phase of acid exposure. Any reduction in oxidative processes such as those involved in hepatic regulation of carbohydrate, protein and fat metabolism would reduce overall bodily needs for oxygen at a critical time when trout suffer from oxygen lack stemming from acid induced gill damage.

The fact that the decreased RNA values after subacute exposure are not significantly different from those obtained after acute exposure indicate that the decrease in RNA occurs early and is a sustained response to acid exposure. Since nuclear RNA content is considered a reflection of cytoplasmic protein synthesis, it would follow that a curtailment of protein synthesis is among the first manifestations of liver dysfunction following acid exposure. A reduction in nuclear regulatory aspects of protein synthesis in liver cells would not necessarily result in reduced synthesis of cytoplasmic structural proteins since the cells are not damaged but metabolically depressed. Thus, the fact that the histological appearance of liver cells is the same in acid exposed and control trout does not signify these cells have comparable levels of metabolic activity. The absence of any evidence of nuclear or cytoplasmic pathology simply indicates that there is no cell deterioration associated with either acute or subacute exposure to acidified water.

Quantitative analyses of Feulgen DNA (F-DNA) and fast green histone (FG-histone) content confirmed that acid exposure of trout does not cause any nuclear damage (DNA loss) nor change the rate of mitotic activity in liver cells. Comparison of the F-DNA or FG-histone in each fish group reveal similar profiles which probably reflects the existence of a close functional relationship between DNA and histones in fish liver. Bloch and Godman (1955) were among the first to demonstrate that an increase in F-DNA occurs coincidentally with an increase in FG-histone in mitotic cells of mammalian liver. They concluded that the synthesis of DNA and histones proportionally parallel each other.

Quantitative analyses of F-DNA were initiated to investigate the functional relationship between DNA and RNA. On the basis of earlier work performed in this laboratory (Pearson, 1970) it was anticipated that a negative shift of small magnitude might be seen in F-DNA corresponding to the decrease in nuclear RNA noted in experimental fish. Such a shift would represent a qualitative rather than a quantitative change in DNA and would reflect the inaccessibility of some portion of DNA for Feulgen dye binding due to compaction or coiling of that portion of the DNA. This rationale stems from a number of recent studies showing that the synthesis of RNA is directly proportional to the availability of sites on DNA. Of importance is the fact that the Feulgen stain in many instances seems to bind somewhat proportionally, but not exclusively, to the same sites which are available for templating RNA. It is generally agreed that only 5 per cent of the genome serves as template for RNA synthesis. The anticipated changes in F-DNA values, due to the unavailability of binding sites, would thus be small. In view of these considerations, it is not too surprising to find that Feulgen dye binding was not altered in acid exposed trout liver tissue.

Although no change in total histone content was noted, quantitative histochemical changes in nuclear (lysine + tyrosine) and arginine were found associated with decreased nuclear RNA levels in experimental fish. It was shown by electrophoretic studies of acid-extracted histones that these changes could be attributed to an increase in lysine rich and a decrease in arginine rich histones. It should be further noted that the increase in lysine rich histone occurs at the expense of the arginine rich histone fraction which probably explains why the amount of total histone (i.e., FG-histone) remains unaltered. These data lend indirect support to the contention that lysine rich histones are effective inhibitors of DNA-dependent RNA synthesis (Huang et al., 1964).

The utility of eosin-fast green staining technique for evaluating functional metabolic changes in the rat liver was established in a previous study (Crissman, 1968). It was found that liver nuclei bordering the portal vein exhibit an increase in eosinophilia following exposure of rats to oxygen lack. Failure to demonstrate this in liver cells of acid exposed trout might be due to the fact that fish liver is not organized into small lobules as are found in mammalian liver where certain cells are much less oxygenated than others.

Staining of histone fractions in polyacrylamide gels with eosin-fast green may prove to be a useful application of the staining method. Histone fractions varied in their affinities for eosin and fast green as reflected by the E_{640}/E_{500} ratios obtained by spectrophotometric analyses. Claypool and Bloch (1967) have shown that with a decreased RNA synthesis there is a decreased E_{640}/E_{500} ratio in nuclei stained with eosin-fast green. In the present study the E_{640}/E_{500} ratio was lower in all three histone fractions of experimental fish when compared to controls although no other observable differences were noted. Further work would be needed, however, to establish that the acquisition of eosin in histone fractions, as reflected in a decreased E_{640}/E_{500} ratio, could be used as an index of depressed RNA synthesis.

At present, there is good evidence that acetylation of histones represents one of the earliest chemical events in the process of gene activation for RNA synthesis. This is based mainly on studies of rat liver preparations. The role of histone acetylation in fish liver is not known; however, the data in this study show that histone acetylation probably occurs in fish liver in much the same manner as in rat liver. Acetate is rapidly incorporated into histones at a constant rate over a one hr interval. No differences in the acetylation patterns were noted between experimental and control fish indicating that histone acetylation in trout liver is not altered during the first hr of acute acid exposure.

Data obtained on lipid synthesis demonstrate that acetate is rapidly incorporated into fish liver lipids. Both control and experimental fish were found to synthesize and release lipids such as fatty acids and triglycerides which are generally used as energy sources. These exploratory tests merely indicate that the liver is not totally incapacitated with respect to lipid synthesis during early periods of acid exposure.

Supplemental histochemical analyses of glycogen and lipid content in fish liver again attest to the value of histochemical methodology in detecting metabolic alterations. These studies show that energy stores in the form of glycogen and lipids are not markedly depleted in liver during acid exposure. This probably reflects an overall reduction in utilization of energy by bodily tissues. Again this provides evidence that liver cells are not damaged but simply metabolically inhibited during acid exposure.

Summary. Three groups of hatchery-reared brook trout (*Salvelinus fontinalis*) were exposed to different levels of acidity over various time periods. One group of trout was exposed to near toxic levels of acidity for 5 d where pH was maintained at 4.0 for 4 d and lowered to 3.5 on day five (acute exposure). The second group of trout was continuously exposed to sublethal levels of acidity (pH 4.7) for 28-30 d (subacute exposure). The final group of hatchery trout was exposed to acidity under field conditions (pH 4.8 for 24-48 hr). Control groups were maintained in each case.

A portion of liver from each fish was fixed in neutral formalin, paraffin embedded, sectioned at 6 microns and stained by the Feulgen or alkaline fast green method for DNA and histone analyses. Some tissue sections were double-stained for (lysine + tyrosine) and for arginine, by the fluorodinitrobenzene (FDNB) and the Sakaguchi reactions. Other tissues were double stained for (lysine + tyrosine) and for RNA by the FDNB and the azure B methods. Sections stained with azure B were pretreated with deoxyribonuclease. Eosin-fast green staining for differentiating nuclear histone types was also employed.

Cytophotometric analyses were performed on liver parenchymal nuclei from each stained group. These studies indicate that no quantitative change occurs in total Feulgen-DNA or fast green-histone after acid exposure. However, analyses of nuclear RNA and two amino acid residues (lysine and arginine) of histones revealed alterations in these components after both acute and subacute exposure of trout. The data showed an increase in the nuclear (lysine + tyrosine)/arginine molar ratio associated with a decrease in nuclear RNA. Since lysine rich histones are effective inhibitors of DNA dependent RNA synthesis, one would suspect a decrease in RNA synthesis concomitant with an increase (lysine + tyrosine) content. This proved to be the case.

Data obtained from electrophoresis of histones extracts substantiate the histochemical data. These studies show a quantitative increase in lysine rich histone with a proportional decrease in arginine rich histone in acid exposed trout.

Spectrophotometric analyses of eosin-fast green stained nuclei reveal a peak at 620 nm in eosinophilic liver nuclei of control and experimental fish which is not observed in eosinophilic rat liver nuclei. It was speculated that the 620 nm peak reflects some differences in molecular configuration of fish liver histone as compared to rat liver. No significant differences were noted with respect to the number of eosinophilic nuclei in acid exposed trout or control trout.

Supplemental information on liver lipid and glycogen levels was obtained following semiquantitative histochemical staining by the Sudan Black B or the periodic acid-Schiff method, respectively. These studies reveal that the levels of these metabolites in liver are not markedly changed after acid exposure. Supplemental data obtained from ^{14}C-acetate tracer studies showed no alterations in the pattern of histone acetylation or lipid synthesis during the first hr of acute acid exposure. However, insufficient data were obtained to draw conclusions regarding rates of utilization of these liver metabolites during acid exposure. The data did, however, reveal that acid exposure of trout does not totally incapacitate metabolic mechanisms in liver.

It was suggested that decreased liver function may be a contributing factor responsible for the reduced tolerance of trout to acid polluted streams.

A schematic summary which related acid induced liver changes to other tissue responses described in previous subsections III, IV and Vi is shown in Figure 32. Included in this figure are some of the histochemical tests which can serve as indices of acid stress.

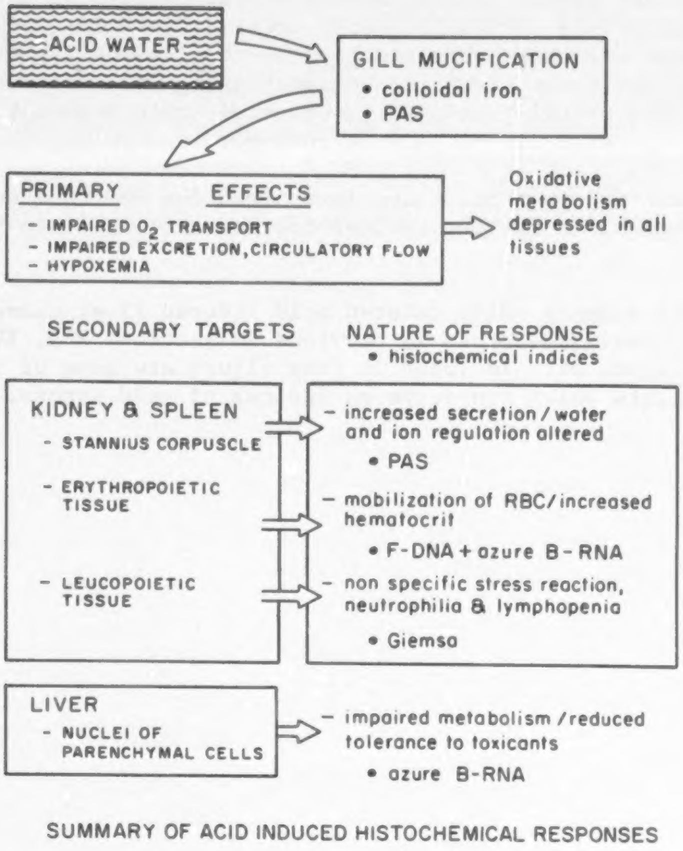
ACID WATER
GILL MUCIFICATION
• colloidal iron
• PAS
PRIMARY EFFECTS
- IMPAIRED O2 TRANSPORT
- IMPAIRED EXCRETION, CIRCULATORY FLOW
- HYPOXEMIA
Oxidative metabolism depressed in all tissues
SECONDARY TARGETS
NATURE OF RESPONSE
• histochemical indices
KIDNEY & SPLEEN
- STANNIUS CORPUSCLE
- ERYTHROPOIETIC TISSUE
- LEUCOPOIETIC TISSUE
- increased secretion / water and ion regulation altered
• PAS
- mobilization of RBC/increased hematocrit
• F-DNA + azure B-RNA
- non specific stress reaction, neutrophilia & lymphopenia
• Giemsa
LIVER
- NUCLEI OF PARENCHYMAL CELLS
- impaired metabolism / reduced tolerance to toxicants
• azure B-RNA
SUMMARY OF ACID INDUCED HISTOCHEMICAL RESPONSES

Figure 32. Schematic outline of hemal, erythrocytic and hepatic responses in fish exposed to acid polluted water

SECTION VII

ACKNOWLEDGMENTS

A large number of faculty and students in the Department of Biology have contributed to this project. The grant director and author of this report was Dr. Adam Anthony, Professor of Zoology and Chairman of the Physiology Program. Project coinvestigators were Drs. E. L. Cooper, C. D. Therrien, R. B. Mitchell and W. H. Neff.

This work was carried out in the Physiology and Histochemistry Laboratories, Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802.

The research was supported in part by Federal Water Pollution Control Administration grant USDI 18050 DXJ and also The Pennsylvania Agricultural Experiment Station.

We wish to acknowledge the contributions of Dr. Robert L. Butler of the Pennsylvania Cooperative Fisheries Unit and Dr. Arthur Bradford, Chief Fisheries Division of the Pennsylvania Fish Commission in the procurement of fish and apparatus needed for maintenance of fish in the laboratory. We also wish to thank Dr. Quenton Pickering and Dr. William A. Brungs of the Newtown Laboratory at Cincinnati, Ohio for helping us with setting up the gravity flow diluter system used in our work.

Particular appreciation is extended to Dr. James M. McKim III of FWPCA National Water Quality Laboratory in Duluth, Minnesota, who acted as Project Officer of this grant and arranged for the shipment of fish tissue specimens from research projects dealing with chronic exposures to toxicants at the Duluth and Newtown FWPCA laboratories.

SECTION VIII

REFERENCES

Alfert, M. and I. I. Geschwind. 1953. A selective staining method for basic proteins of cell nuclei. Proc. Nat. Acad. Sci. 39: 991-999.

Bloch, D. P. 1966. Histone differentiation and nuclear activity. Chromosoma. 19:317-339.

Bloch, D. P. and S. Brack. 1964. Evidence for cytoplasmic synthesis of nuclear histones during spermiogenesis in the grasshopper, *Chortophaga viridifasciata* (de Geer). J. Cell Biol. 22:327-340.

Bloch, D. P. and G. C. Godman. 1955. A microspectrophotometric study of the synthesis of desoxyribonucleic acid and nuclear histone. J. Biophys. Biochem. Cytol. 1:17-28.

Bloch, D. P. and H. V. C. Hew. 1960. Schedule of spermatogenesis in the pulmonate snail, *Helix aspersa*, with special reference to histone transition. J. Biophys. and Biochem. Cytol. 7:515.

Bonner, J., G. R. Chalkley, M. Dahmus, D. Fambrough, F. Fujimura, R. C. Huang, J. Huberman, R. Jensen, K. Marashige, H. Ohlenbusch, B. Olivera, and J. Widholm. 1968. Preparation of nucleoproteins: isolation and characterization of chromosomal nucleoproteins. *In* L. Grossman and K. Muldave (eds). Methods of enzymology, Vol. III, Nucleic acids, Part B, Academic Press, New York.

Carpenter, K. E. 1927. The lethal action of soluble metallic salts on fishes. Brit. J. Exp. Biol. 4:378-390.

Chester-Jones, I., I. E. Henderson and D. G. Butler. 1966. Water and electrolyte flux in the European eel (*Anguilla anguilla*). Arch. Anat. Microscop. Morphol. Exptl. 54:453-469.

Conte, F. P. 1969. Salt secretion. *In* W. S. Hoar and D. J. Randall (eds.), Fish physiology, Vol. I, 241-292.

Crissman, H. A. 1968. Eosin-fast green staining of nuclear histones in liver cells of normal and hypoxic rats. M.S. Thesis, The Pennsylvania State University.

Crissman, H. A. and C. D. Therrien. 1970. Cytophotometric and electrophoretic analyses of DNA and histones in liver parenchymal nuclei of acid-exposed brook trout, *Salvelinus fontinalis*. Pa. Acad. Sci. 44:in press.

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Deitch, A. D., D. Wagner and R. M. Richart. 1968. Conditions influencing the intensity of the Feulgen reaction. J. Histochem. Cytochem. 16:371-379.

Feulgen, R. and H. Rossenbeck. 1924. Mikroskopischchemischer nachweis einer nuclein saure von typus der thymonucleinsaure und die darauf beruhende elektive farbund von zellkernen in mikroskopischen praparaten. Z. Physiol. Chem. 135:203-248.

Flax, M. H. and M. Himes. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. Physiol. Zool. 25:297.

Folch, J., M. Lees and G. H. Sloane Stanley. 1957. A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Fontaine, M. 1964. Corpuscles de Stannius et regulation ionique (Ca, K, Na) du milieu interieur de l'anguille (Anguilla anguilla L.) C. R. Acad. Sci. Paris. 259:875-878.

Fujiya, M. 1961. Effects of Kraft pulp mill wastes on fish. Journ. WPCF. Vol. 33, 9:968-977.

Garcia, A. M. 1967. On Feulgen-deoxyribonucleic acid haploid and diploid values. J. Histochem. Cytochem. 15:778.

Garcia, A. M. 1969. Studies on deoxyribonucleic acid in leucocytes and related cells of mammals. VI. The Feulgen-deosyribonucleic acid content of rabbit leucocytes after hypotonic treatment. J. Histochem. Cytochem. 17:47-55.

Garcia, A. M. 1969b. Studies on deoxyribonucleic acid in leucocytes and related cells of mammals. VII. The fast green histone content of rabbit leucocytes after hypotonic treatment. J. Histochem. Cytochem. 17:475-479.

Gledhill, B. L. 1966. Studies on the DNA content, dry mass and optical area of bull spermatozoal heads during epididymal maturation. Acta Vet. Scand. 7:131-142.

Hanke, W. and I. Chester-Jones. 1966. Histological and histochemical studies on the adrenal cortex and corpuscle of Stannius of the european eel (Anguilla anguilla). Gen. and Comp. Endocrinol. 7: 166-178.

Hay, E. D. and J. P. Revel. 1963. The fine structure of the DNP component of the nucleus. J. Cell Biol. 16:29-51.

Hesser, E. F. 1960. Methods for routine fish hematology. Progr. Fish Cult. 22:164-171.

Huang, R. C., J. Bonner and K. Murray. 1964. Physical and biological properties of soluble nucleohistones. J. Molec. Biol. 8:54-64.

Humason, G. L. 1967. Animal tissue techniques. 2nd ed. W. H. Freeman Co., San Francisco, 569 pp.

Jackowska, S. 1956. Morphologie et nomenclature des cellules du sang des teleosteens. Rev. D'Hemat. 11:519-539.

Jacobson, L. O. and M. Doyle (eds.). 1962. Erythropoiesis. Grune and Stratton, New York., 399p.

Leuchtenberger, C. and F. Schrader. 1952. Variation in the amounts of desoxyribose nucleic acid (DNA) in cells of the same tissue and its correlation with secretory function. Proc. Nat. Acad. Sci. (USA). 38:99-105.

Littau, V. C., V. G. Alfrey, J. H. Frenster and A. E. Mirsky. 1964. Active and inactive regions of nuclear chromatin as revealed by electron microscope autoradiography. Proc. Nat. Acad. Sci. 52: 93-100.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Mayall, B. H. 1967. Variability in the stoichiometry of deoxyribonucleic acid stains. J. Histochem. Cytochem. 15:762-763.

Mayall, B. H. 1969. Deoxyribonucleic acid cytophotometry of stained human leucocytes. I. Differences among cell types. J. Histochem. Cytochem. 17:249-255.

McKim, J. M., G. M. Christensen and E. P. Hunt. 1970. Changes in the blood of brook trout (Salvelinus fontinalis) after short-term and long-term exposure to copper. J. Fish. Res. Bd. Canada 27: 1883-1889.

Moss, B. and V. M. Ingram. 1968. Hemoglobin synthesis during amphibian metamorphosis. I. Chemical studies on the hemoglobins from the larval and adult stages of Rana catesbiana. J. Mol. Biol. 32:481-492.

Mount, D. I. and W. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water. Res. 1:21-29.

Mowry, R. W. 1963. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins with revised directions for the colloidal iron stain, the use of alcian blue 8GX and their combination with periodic acid-Schiff reaction. Ann. N. Y. Acad. Sci. 106:402-423.

Noeske, K. 1971. Discrepancies between cytophotometric Feulgen values and deoxyribonucleic acid content. J. Histochem. Cytochem. 19:169-174.

Ornstein, L. 1952. The distributional error in microspectrophotometry. Lab. Invest. 1:250-265.

Ostroumova, I. N. 1970. Properties of the blood of trout during adaptation to different oxygen and salt contents of the water. p. 24-33. In T. I. Privol'nev (ed.). Fish Physiology in acclimatization and breeding, Israel Program for Scientific Translations Ltd., Jerusalem.

Packer, R. and W. A. Dunson. 1970. Effects of low environmental pH on blood pH and sodium balance of brook trout. J. Exper. Zool. 174:65-72.

Palau, J. and J. A. V. Butler. 1966. Trout-liver histones. Biochem. J. 100:779-783.

Patau, K. 1952. Absorption microphotometry of irregular shaped objects. Chromosoma. 5:341-362.

Pearson, P. F. 1970. Cytophotometric and biochemical analysis of thyroid cell metabolism in hypoxia exposed rats. Ph.D. Thesis, The Pennsylvania State University, 67 pp.

Plonka, A. C. 1971. Histochemical and histological analyses of gills and Stannius corpuscles in acid exposed brook trout. Ph.D. Thesis, The Pennsylvania State University, 66 pp.

Plonka, A. C. and W. H. Neff. 1969. Mucopolysaccharide histochemistry of gill epithelial secretions in brook trout (Salvelinus fontinalis) exposed to acidic conditions. Pa. Acad. Sci. 43:53-56.

Roels, H. 1954. Cell activity and deoxyribonucleic acid content of the nuclei of the thyroid gland of the white rat. Nature (London). 174:514-515.

Roels, H. 1966. Metabolic DNA - a cytochemical study. Int. Rev. Cytol. 19:1.

Schiffman, R. A. and P. O. Fromm. 1959. Chromium-induced changes in the blood of rainbow trout, *Salmo gairdneri*. Sewage Ind. Wastes. 31:205-211.

Swift, H. 1950. The desoxyribose nucleic acid content of animal nuclei. Physiol. Zool. 23:169-198.

Swift, H. 1964. The histones of polytene chromosomes. p. 169-181. *In* J. Bonner and P. Ts's (eds.). The Nucleohistones, Holden-Day, Inc., San Francisco.

Swift, H. and E. Rasch. 1956. Microspectrophotometry with visible light. *In* G. Oster and A. W. Pollister (eds.), Physical techniques in biological research, Academic Press, New York. pp 353-400.

Therrien, C. D. 1967. Cytophotometry of nucleolar and cytoplasmic ribonucleic acid and basic protein in the plasmodial slime mold *Physarum pusillum*. Mycologia 59:757-766.

Vaala, S. S. 1970. Blood pO_2 changes in acid exposed brook trout. Pa. Acad. Sci. 44:in press.

Vaala, S. S. 1971. Erythrocytic indices of stress in brook trout exposed to sublethal levels of acidity. Ph.D. Thesis, The Pennsylvania State University. 65pp.

Vaala, S. S. and R. B. Mitchell. 1970. Blood pO_2 changes in acid exposed brook trout. Pa. Acad. Sci. 44:in press.

Vasil'ev, A. V. 1948. Gematologiya Sel'skokhozyaistvennykh zhivotnykh (Hematology of agriculture animals). Moskva, Sel'khozgiz.

Vaughn, J. C. 1966. The relationship of the "sphere chromatophile" to the fate of displaced histones following histone transition in rat spermiogenesis. J. Cell. Biol. 31:247-278.

Warner, R. E. 1967. Bioassays for microchemical environmental contaminants. Bull. World Health Organ. 36:181-207.

Weinreb, E. L. 1958. Studies on the histology and histopathology of the rainbow trout. *Salmo gairdneri irideus*. I. Hematology: Under normal and experimental conditions of inflammation. Zoologica. 43:145-154.

Weinreb, E. L. 1959. Studies on the histology and histopathology of the rainbow trout, *Salmo gairdneri irideus*. II. Effects of induced inflammation and cortisone treatment on the digestive organs. Zoologica. 44:45-52.

Weinreb, E. L. 1963. Studies on the fine structure of teleost blood cells. I. Peripheral blood. Anat. Rec. 147:219-238.

Weinreb, E. L. and S. Weinreb. 1969. A study of experimentally induced endocytosis in a teleost. I. Light microscopy of peripheral blood cell responses. Zoologica. 54:25-34.

Westfall, B. A. 1945. Coagulation film anoxia in fishes. Ecology. 26:283-287.

Wied, G. L. (ed.). 1966. Introduction to quantitative cytochemistry. Vol. I, Academic Press, New York. 623 pp.

Wied, G. L. and G. F. Bahr. 1970. Introduction to quantitative cytochemistry. Vol. II, Academic Press, New York. 551 pp.

SECTION IX

PUBLICATIONS

Master of Science Theses

Plonka, A. C. 1969. Mucopolysaccharide histochemistry of gill epithelial secretions in brook trout (Salvelinus fontinalis) exposed to acidic conditions, The Pennsylvania State University. 30p.

Weyandt, T. B. 1970. Gas chromatographic analysis of muscle and liver lipids of brook, brown and rainbow trout, The Pennsylvania State University. 41p.

Dively, J. L. 1970. Gas chromatographic analysis of muscle lipids in normal and acid exposed brook trout, The Pennsylvania State University. 31p.

Trusal, L. R. 1970. Feulgen-DNA cytophotometry of adipose cells, erythrocytes, fibroblasts and spermatogenic cells of brook trout, The Pennsylvania State University. 50p.

Mudge, J. E. 1970. Histology and DNA cytophotometry of the pancreas in acid exposed brook trout, The Pennsylvania State University. 48p.

Dolsky, M. F. 1971. Hemal changes in acid exposed fathead minnows and longnose dace, The Pennsylvania State University.

Ph.D. Dissertations

Plonka, A. C. 1971. Histochemical and histological analyses of gills and Stannius corpuscles in acid exposed brook trout, The Pennsylvania State University. 68p.

Vaala, S. S. 1971. Erythrocytic indices of stress in brook trout exposed to sublethal levels of acidity, The Pennsylvania State University. 92p.

Crissman, H. A. 1971. Cytochemistry of DNA, RNA and histones in liver nuclei of acid exposed brook trout, The Pennsylvania State University. 73p.

Papers

Crissman, H. A. and C. D. Therrien. 1969. Cytochemical analysis of liver histones in acid exposed brook trout, Salvelinus fontinalis. Proc. Pa. Acad. Sci. 43:49-52.

Plonka, A. C. and W. H. Neff. 1969. Mucopolysaccharide histochemistry of gill epithelial secretions in brook trout exposed to acid pH. Proc. Pa. Acad. Sci. 43:53-55.

Vaala, S. S., R. B. Mitchell and A. Anthony. 1969. Cytophotometric studies of DNA in circulating erythrocytes of brook trout exposed to acid pH. Proc. Pa. Acad. Sci. 43:191-194.

Crissman, H. A. and C. D. Therrien. 1970. Cytochemical and biochemical analyses of DNA and histones in liver parenchymal nuclei of acid exposed brook trout. Proc. Pa. Acad. Sci. 44:

Mudge, J. E. and W. H. Neff. 1970. Microscopic anatomy of the pancreas of the brook trout. Proc. Pa. Acad. Sci. 44:

Vaala, S. S. and R. B. Mitchell. 1970. Blood P_{O_2} changes in acid exposed brook trout. Proc. Pa. Acad. Sci. 44:

Neff, W. H., L. R. Trusal and A. Anthony. 1970. Feulgen-DNA cytophotometry of erythrocytes, fibroblasts and adipose cells of brook trout. Anat. Rec. 166(2):354.

Plonka, A. C. R. B. Mitchell and A. Anthony. 1971. Quantitative cytophotometry of PAS positive material in Stannius corpuscles of brook trout exposed to acidic water. Proc. Pa. Acad. Sci. 45: in press.

Dively, J. L., G. Porter and A. Anthony. 1971. Fatty acid composition of muscle lipids of normal and acid exposed brook trout. Proc. Pa. Acad. Sci. 45: in press.

Vaala, S. S., R. B. Mitchell and A. Anthony. 1971. Erythrocytic changes associated with subacute acid exposure of brook trout. Proc. Pa. Acad. Sci. 45: in press.

SECTION X

GLOSSARY

acidophilia - cytoplasmic affinity for acid dyes.

AU - arbitrary units. The total amount of absorbing material in a nucleus or cell is generally expressed in arbitrary photometric units and is a function of the measured optical density (extinction), the specimen area and the extinction coefficient of the dye used.

azure B-RNA - complex of ribonucleic acid stained with azure B; nuclear and cytoplasmic RNA can be measured cytophotometrically following azure B staining provided DNA is first enzymatically removed using DNAase.

basophilia - nuclear and cytoplasmic affinity for basic dyes. The substance largely responsible for basophilic staining in the cytoplasm is ribonucleoprotein.

compact or condensed chromatin - refers to those segments of chromosomes that are tightly coiled in the interphase nucleus; are visible as chromatin particles with light microscopy; also referred to as heterochromatin although this term has certain connotations to cytogeneticists which make use of the prefixes hetero- and eu- less suitable for describing chromatin stainability or its appearance.

dispersed chromatin - refers to chromatin of those segments of chromosomes that are extended and with light microscopy are invisible in the interphase nucleus; also referred to as diffuse chromatin or euchromatin by histochemists.

DNAase - desoxyribonuclease, a purified enzyme available commercially and used for preparing control slides in F-DNA analyses and in cytophotometric analysis of RNA.

F-DNA - Feulgen stained desoxyribonucleic acid complex. The Feulgen reaction utilizes the Schiff reaction for aldehydes but involves the use of mild hydrolysis (under standard conditions). This liberates aldehydes from the D-2-deoxyribose of DNA but not those from the ribose of RNA nor any other sugars.

H and E - hematoxylin (a basic dye) plus eosin (an acid dye) staining represents the most common combination of stains used for routine microscopic analyses of tissues.

hemopoiesis - formation of white blood cell or leucoid elements (leucopoiesis) and red blood cell or erythroid elements (erythropoiesis). In teleosts, erythropoiesis occurs primarily in renal hemopoietic tissue and leucopoiesis in the spleen although both organs manufacture WBC and RBC.

histones - relatively simple basic proteins. These are the most abundant and most important proteins in chromosomes. Histone levels show a quantitative correlation with DNA content in many cell types.

microspectrophotometry - application of spectroscopic methods for the quantitative analysis of chemical constituents in tissue cells. Synonyms used include: cytophotometry, microphotometry and analytical microscopic histochemistry.

PAS - the periodic acid Schiff method is a two step histochemical procedure for mucopolysaccharides, first utilizing periodic acid to release aldehydes from glucose residues of carbohydrates and then treating sections with Schiff reagent (leucofuchsin) which reacts with the aldehydes to form a purple dye (basic fuchsin).

RNAase - ribonuclease, a purified enzyme available commercially and used for preparing control slides in RNA analyses.

S-G_2 stage - the DNA duplication or synthesis (S) stage and post-duplication (G_2) stage of interphase represent the intervals of the mitotic cycle where one would expect nuclei to contain interclass (3C) and tetraploid (4C) contents of F-DNA.

SECTION XI

PERSONNEL

Staffing: Most of the work described was performed by graduate students supervised by the following staff members in the Department of Biology. Dr. Adam Anthony, Professor of Zoology and Principle Investigator; Dr. E. L. Cooper, Professor of Zoology, Coinvestigator; Dr. C. D. Therrien, Associate Professor of Biology, Coinvestigator; Dr. R. B. Mitchell, Assistant Professor of Biology and Dr. W. H. Neff, Assistant Professor of Biology. Mrs. Athleen J. Stere and Charles Wagner were employed on a half time basis as Research Assistant and Fisheries Research Technician, respectively. Brief biographical sketches of graduate students, most of whom held traineeships which permitted them to participate in this research as part of their graduate training are given below with their interval of association with this project in parenthesis.

Harry A. Crissman, M.S., 1968, Ph.D., 1971, The Pennsylvania State University. He held an NDEA fellowship from 9-68 to 8-71. Currently has accepted a research position at the Los Alamos Radiation Laboratory, New Mexico effective August, 1971. (3-68 to 5-71).

James L. Dively, M.S. 1970 in Physiology, The Pennsylvania State University. Currently holds an NDEA fellowship and is a doctoral candidate in Physiology. (3-68 to 5-71).

Mary F. Dolsky, M.S. 1971 in Physiology, The Pennsylvania State University. Was awarded an NSF traineeship which she has resigned to enable her to accept a FWPCA research fellowship effective September, 1971. (3-69 to 5-71).

James E. Mudge, M.Ed, 1969; M.S. 1970, The Pennsylvania State University. Currently a doctoral candidate in Physiology and holds a teaching assistantship in biology. (10-69 to 5-71).

Andria C. Plonka, M.S., 1969; Ph.D. 1971, The Pennsylvania State University (3-68 to 3-71).

Lynn R. Trusal, M.Ed., 1970, M.S., 1970, The Pennsylvania State University. Was admitted as doctoral candidate in Physiology and was drafted. Currently a 2nd lieutenant teaching physiology at the Medical Field Service School, Fort Sam Houston, Texas. (9-68 to 8-70).

Sharon S. Vaala, M.S. 1968, Ph.D. 1971, The Pennsylvania State University. Held an NSF traineeship 1967-1971. Currently has accepted a position in the Department of Anatomy, University of Rochester Medical School, Rochester, New York. (3-68 to 5-71).

Timothy B. Weyandt, M.S. 1970, The Pennsylvania State University. Held a NASA traineeship 1969-1970. Admitted as doctoral candidate and drafted. Currently teaching in Medical Field Service School, Fort Sam Houston, Texas. (9-68 to 9-70).

SECTION XII

APPENDIX

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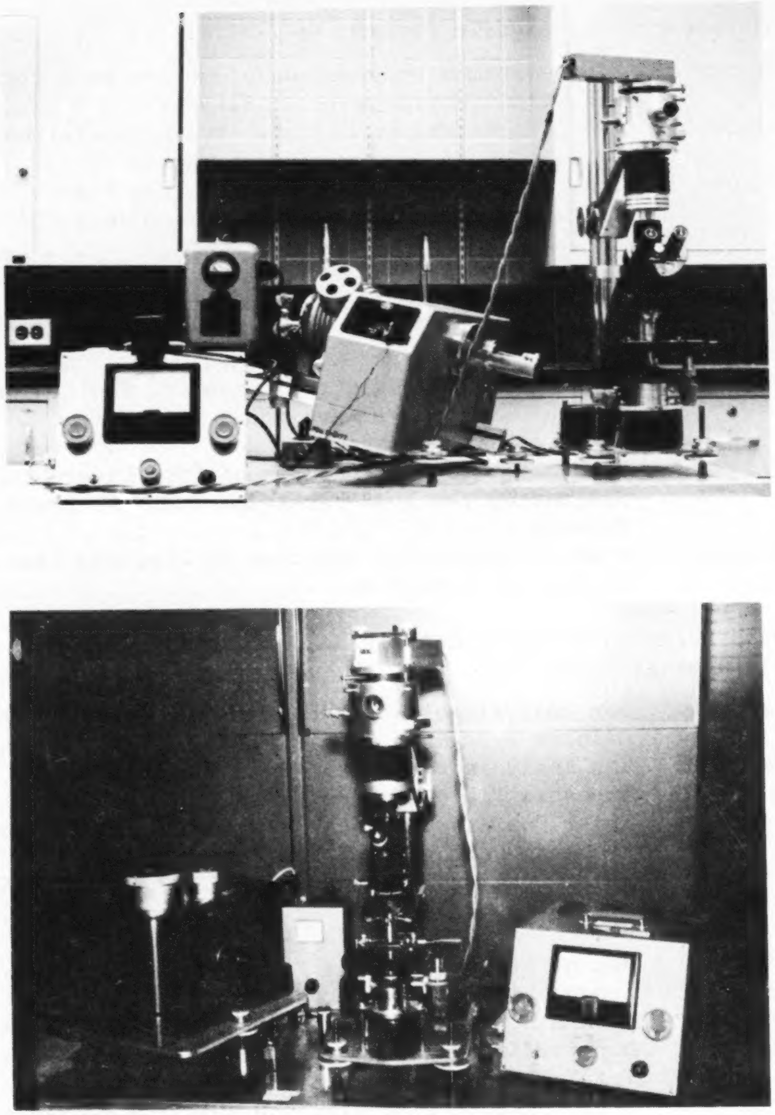


Figure 1. Single Beam Microspectrophotometers used for Analytical Histochemical Analyses.

APPENDIX

A. MICROSPECTROPHOTOMETRIC PROCEDURES

Cytophotometric measurements were made with two specially constructed microspectrophotometers (Figure 1), each consisting of the following basic components: (1) Photovolt power supply and meter; (2) Leitz or Zeiss monochromator and tungsten light source; (3) Leitz Ortholux microscope with 20x, 0.4 N.A. quartz objective used as a condenser and 95x, 1.32 N.A. objective lens; and (5) photodetector RCA 1P21 photomultiplier. Validation checks of cytophotometric methodology were also performed using a custom built, two beam, UV-visible recording microspectrophotometer (Figure 2).

Relative amounts of nuclear DNA in various cell types were determined using the two wavelength method of microspectrophotometry. The two wavelength method was introduced simultaneously by Ornstein (1952) and Patau (1952) as an application of the Beer-Lambert Law. The procedure corrects for distributional error for a wide variety of chromophore distributions and therefore is useful when the material being measured is irregular or heterogeneous.

Objects in a photometric field are measured at two wavelengths predetermined to give an extinction ratio of 2:1 for the chromophore under investigation. Appropriate mathematical manipulation of transmittance divides the field into absorbing molecules and nonabsorbing components. An estimate of absorbing molecules depends upon a linear relation between extinction and concentration of dye molecules. The procedure involves selection of two wavelengths (λ_1 and λ_2) from an absorption curve of a homogeneous area such that the extinction at one wavelength (E_1) equals half the extinction (E_2) at the other to give $2 E_1 = E_2$. Under these conditions, $E_1 = \log I_o/I_s$ at λ_1, and $E_2 = \log I_o/I_s$ at λ_2. I_o represents intensity of background or incident light, while I_s is the intensity of the incident beam after being transmitted through a photometric field containing the specimen. For Feulgen-DNA cytophotometry, one has to first standardize the hydrolysis time which can vary depending upon the tissue, fixation or type of tissue preparation (i.e. smear, imprint or section). A typical hydrolysis curve is shown in Figure 3. A representative Feulgen absorption curve for stained red blood cells is shown in Figure 4.

From measures of I_o and I_s at both wavelengths, transmissions T_1 and T_2 are determined and the following calculations performed.

$$T_1 = I_s/I_o \text{ at } \lambda_1 \qquad T_2 = I_s/I_o \text{ at } \lambda_2$$

$$L_1 = 1 - T_1 \qquad L_2 = 1 - T_2$$

$$Q = L_2/L_1 \qquad C = 1/2 - Q \ln 1/Q - 1$$

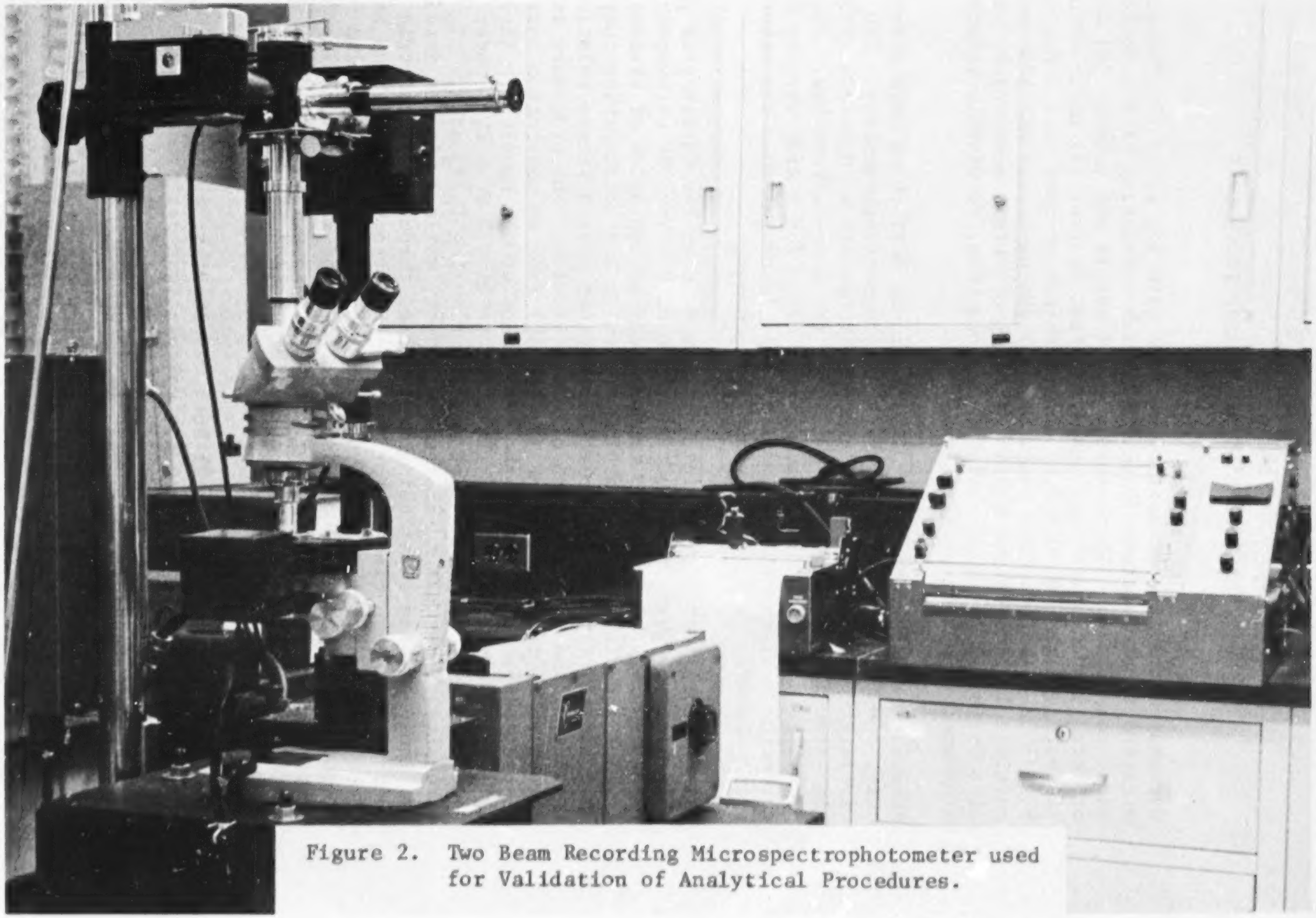


Figure 2. Two Beam Recording Microspectrophotometer used for Validation of Analytical Procedures.

The functions with Q as the parameter have been previously tabulated in the literature by Patau (1952), and the correction factor, C, is listed for corresponding values of Q.

$M = KAL_1C$

$A = r^2$ for relative measurements.

K = a constant (1/e) where e is the extinction coefficient at λ_1 and may be disregarded for relative measurements.

A close approximation of the amount of absorbing molecules within a measured area is given by the value of M, which is presented in arbitrary units (A.U.).

A source of difficulty in the method stems from the involved series of entries and calculations required for each object measured. This represents a potential source of human error and makes the method time consuming and tedious (Mendelsohn, 1966). A Fortran 4 program (Figure 5) was therefore prepared and all calculations computed by an IBM 360-67 computer. Data cards were prepared and supplied with five values in the following order: the aperture diameter of upper viewing tube (circular aperture diameter selected to enclose the image of the object to be measured with a minimum amount of surrounding clear area); photometer readings of I_S at λ_2 and λ_1 and photometer readings of I_O at λ_2 and λ_1. The program is designed to calculate and print out values of T_1, T_2, L_1, L_2, Q, C, and M. Final M values, which are the absorbing molecule concentrations in individual nuclei are printed out in order of decreasing magnitude such that the range of values may be easily subdivided and presented in histogram form.

The standard plug method (Swift, 1950) was also employed for cytophotometric analyses when using double staining techniques. Again, two wavelengths must be selected, each representing the maximum extinction for the dye complex under study. After defining a photometric field within each of several homogeneous areas of the specimen, the two wavelengths are selected and extinctions determined as previously described. Representative absorption curves for trout liver nuclei stained by the FDNB/Sakaguchi and FDNB-azure B methods are shown in Figure 6. As is evident, the shapes of spectral curves obtained are quite different from those obtained using a single stain such as alkaline fast green (Figure 7).

Once the proper wavelengths are selected, the establishment of a standard optical plug through a cellular structure is required. In an effort to minimize possible sources of error, measurements at both predetermined wavelengths were made without moving the optical plug. The background was then recorded for each wavelength. To measure the total nuclear (lysine + tyrosine) and arginine, it is first necessary to determine the extinction of an optical plug which passes through the center of each nucleus. The number of dye-absorbing molecules in the plug is equivalent to KEA, where K is a constant and may be

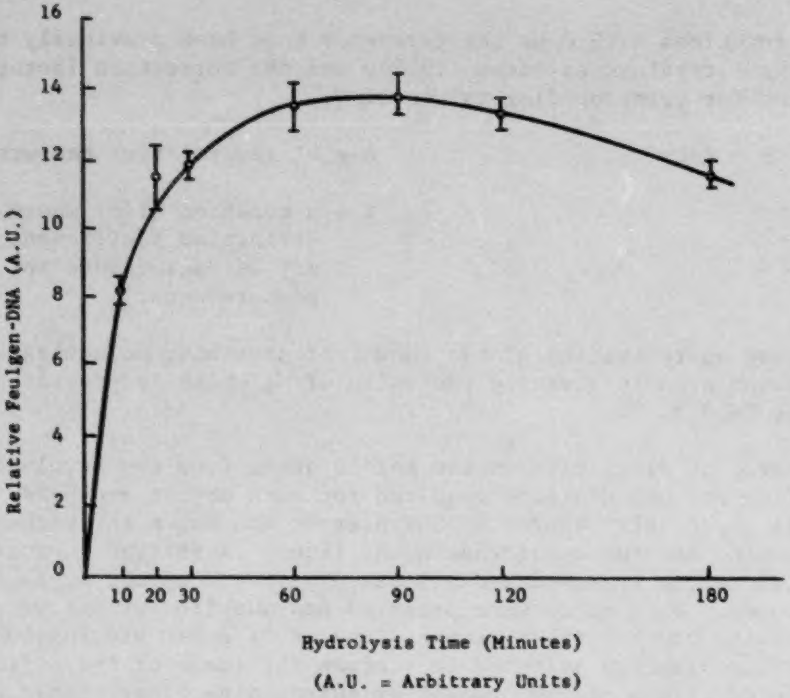


Figure 3. 5 N HCl hydroloysis curve of Feulgen stained erythrocytes

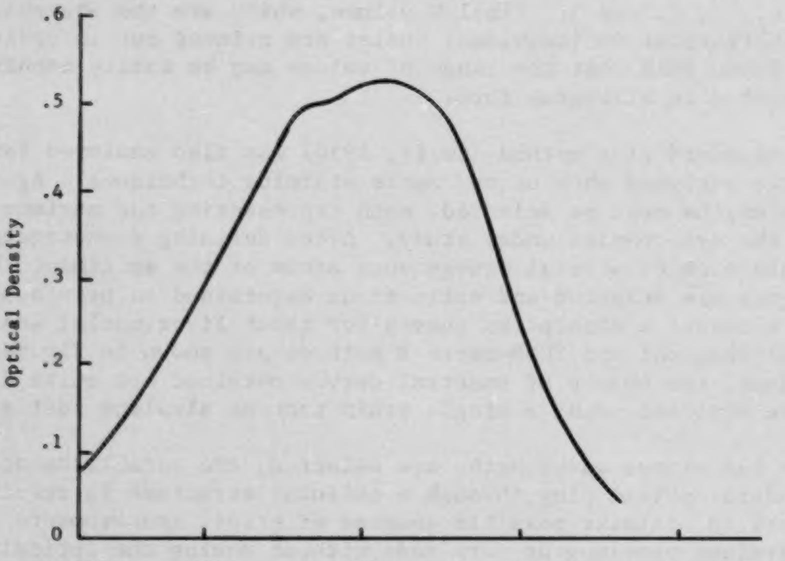


Figure 4. Absorption spectrum of Feulgen stained erythrocytes.

```
PSU-WATFOR *** 5-1
          IMPLICIT REAL *8(A-I,L,M,Q-Z), INTEGER * 4(P)
          LOGICAL * 1 TITLE (80)
          DIMENSION A(1000)
          CALL TRAPS (0,1,100,1,1)
          K = 0
          READ 5, TITLE
    5     FORMAT (80A1)
          PRINT 6, TITLE
    6     FORMAT('1',T25,80A1/'0',T6,'D',T11,'ISAMPB',T19,'ISAMPA',
          T27, 'ISOLIVB',T35,'ISOLVA',T45,'TRANSB',T55,'TRANSA',
          T68,'LB',T78,'LA',T88,'2Q',T98,'C',T108,'M')
    9     READ(5,8, END = 20) D, ISAMPB,ISAMPA,ISOLVB,ISOLVA
    8     FORMAT (5F4.1)
          K = K+1
          A(K) = 0.0D0
          TRANSA = ISAMPA/ISOLVA
          TRANSB = ISAMPB/ISOLVB
          LA = 1.0D0 - TRANSA
          LB = 1.0D0
          IF(LA.GT.0) GO TO 19
          WRITE(6,69)D,ISAMPB,ISAMPA,ISOLVB,ISOLVA
   69     FORMAT('0',5F8.1,T45.' LA IS LESS THAN OR EQUAL TO ZERO)
          GO TO 9
   19     Q = LB/LA
          IF(Q.G.E.2.0D0) GO TO 11
          IF (Q.T.1.0D0) GO TO 10
   11     WRITE(6,12) D,ISAMPB,ISAMPA,ISOLVB,ISOLVA,Q
   12     FORMAT('0',5F8.1,T45,'Q INCORRECT Q = ',D15.8)
          GO TO 9
   10     C = (1.0D0/(2.0D0-Q)*DLOG(1.0D0/(Q-1.0D0)
          M = C*LA*(D/a.0D0)**2
          A(K) = M
          WRITE(6,13)D,ISAMPB,ISAMPA,ISOLVB,ISOLVA,TRANSB,TRANSA,
          LB,LA,Q,C,1M
   13     FORMAT('0',5F8.1, 7F10.3)
          GO TO 9
   20     N = K-1
          DO 17 J = 1,N
          PJ = J-1
          DO 17 P = PJ,K
          IF(A(J).GT.A(P)) GO TO 17
          TEMP = A(J)
          A(J) = A(P)
          A(P) = TEMP
   17     CONTINUE
          WRITE(6,22)
   22     FORMAT('1',T25,'IN DECREASING ORDER THE M VALUES ARE')
          WRITE(6,18) (A(J), J = 1,K)
   18     FORMAT('0',10F10.3)
          STOP
          END
```

Figure 5. Fortran program for Feulgen-DNA calculations using two wavelength method of microspectrophotometry

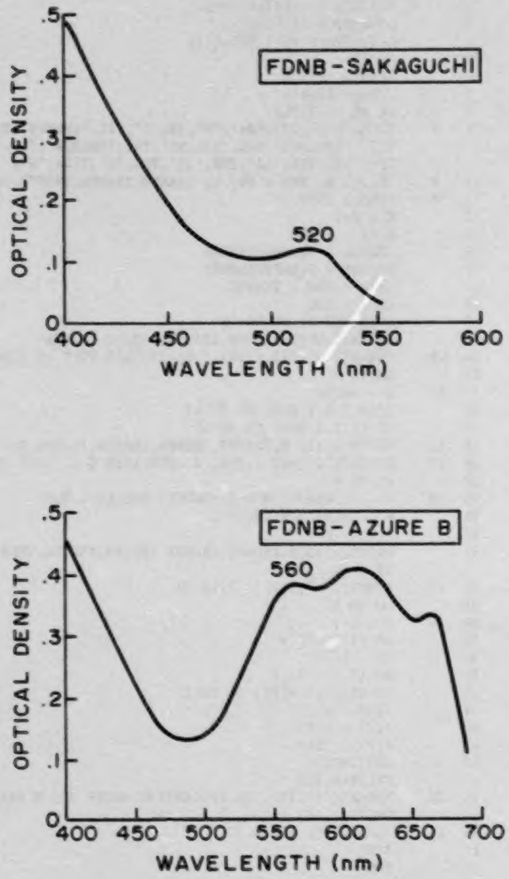


Figure 6. Spectral absorption curves of FDNB-azure B and FDNB-Sakaguchi complexes in trout liver nuclei.

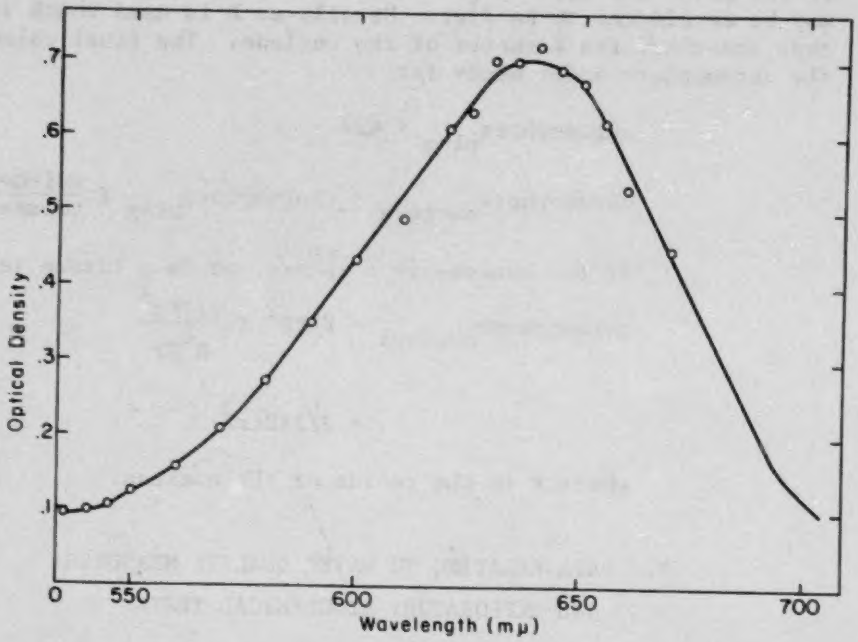


Figure 7. Absorption spectrum for alkaline fast green stained erythrocytes.

disregarded when making relative measurements, E is the extinction determined at the selected wavelength, and A is the area of the plug. Since the area of the plug is πR^2, where R is the radius of the plug, the plug = $KE\pi R^2$. The cylinder obviously contains only a fraction of the total volume of the nucleus; therefore, to obtain a value for the total nuclear volume, the value obtained for the plug must be multiplied by the ratio of nuclear volume over the plug volume. In using the plug method, particular attention should be paid to the distribution of the chromophore under study, since inhomogeneous distribution may lead to serious errors in the measurements. Care should be taken to find entire nuclei in the section, since in tissue, sections are cut at different planes. Care must also be taken that the diameter of the plug is small enough so that the rounded surface of the sphere may be considered to be flat. Usually an R is used which is not more than one-third the diameter of the nucleus. The final calculation for the chromophore under study is:

$$\text{chromophore}_{\text{plug}} = KEA$$

$$\text{chromophore}_{\text{nucleus}} = \text{choromphore}_{\text{plug}} \times \frac{\text{volume nucleus}}{\text{volume plug}}$$

If the nucleus is a sphere, as in a tissue section, then:

$$\text{chromophore}_{\text{nucleus}} = KE\pi R^2 \times \frac{4/3\pi r^3}{R^2 2r}$$

$$= 2/3KE\pi r^2$$

where r is the radius of the nucleus.

B. DATA RELATING TO WATER QUALITY MEASURES AND EXPLORATORY BIOCHEMICAL TESTS

Four tables are included as examples of water analyses run on laboratory aquaria water and field streams (Tables 1, 2, 3 and 4). Figure 8 is a map of Pennsylvania giving locations of stream sites where field investigations were conducted.

Hemoglobin was extracted for 16 acute acid exposed and 16 control trout, and five fractions separated by polyacrilamide electrophoresis. Typical microdensitometer tracings following staining of gels with benzidine dihydrochloride are shown in figure 9. No differences were found between experimental and control blood samples.

Blood histones were extracted, electrophoresed, stained with 0.5 percent amido black for 18 hrs, destained by diffusion in 7 percent acetic acid and scanned using a Gilford densitometer. Figure 10 illustrates typical scans from acid exposed and control trout when too high a concentration

Table 1. Ion analysis of water used in the gravity flow diluter system.

pH	Tank	P	K	Ca	Mg	Mn	Fe	Cu	B	Zn	Na
					(parts per million)						
4.85	1	1.1	3.7	10.5	0.2	.00	.02	.01	.00	.12	34.2
5.86	2	1.3	5.4	5.2	0.2	.01	.05	.01	.01	.08	34.2
6.36	3	1.1	6.2	6.7	0.2	.00	.04	.01	.01	.07	38.8
6.30	4	0.7	6.5	4.8	0.2	.00	.04	.01	.01	.10	35.1
7 02	5	1.2	7.7	4.8	0.2	.00	.07	.01	.02	.12	38.2
3.00	6	0.9	7.3	1.2	0.1	.01	.10	.02	.02	.17	38.6
7.80	7	0.3	4.0	3.4	0.1	.01	.03	.00	.00	.12	33.6
H_2O_d	-	0.9	8.5	1.[illegible]	0.2	.01	.08	.04	.02	.16	0.1
	1	1.6	2.5	7.3	0.1	.00	.01	.00	.00	.08	27 7
	2	3.2	2.7	5.6	0.1	.03	.12	.13	.01	.05	18.5
	3	1.5	9.6	7.2	0.2	.02	.08	.02	.02	.10	33.9
	4	1.7	8.7	6.1	0.2	.01	.07	.01	.02	.07	42.9
	5	1.1	6.2	5.5	0.2	.01	.05	.01	.01	.10	41 8
H_2O_d	6	0.7	6.3	0.9	0.0	.01	.06	.02	.01	.02	0 0
	7	1.6	7.3	3.9	0.2	.01	.07	.02	.02	.27	43.8
					Values too low so absolute levels probably questionable						

Al is too low for proper detection. H_2O_d is a distilled water control.

Table 2. Ion analysis of streams used in acute acid exposure field studies.

Stream	K	Na	Ca	Zn	Mg	Mn	Fe	Cu	B	Al
Upper Three Runs (Acid branch)	7.00	5	7.6	1.6	5.0	5	5	0.06	0.07	5
Upper Three Runs (Below acid branch)	0	1.1	6	0.03	5.3	0.98	0	0	0	0.76
Upper Three Runs (Above acid branch)	0	0.9	2	0	1	0.01	0	0.01	0	0.07
Black Moshannon (Below acid branch)	0	1.3	7	0.05	3.6	0.48	0.02	0.01	0	0.36
Black Moshannon (Above acid branch)	0	0.7	2	0.03	0.02	0.01	0.01	0	0	5

Table 3. Water quality in Black Moshannon Creek 29 April 1970 and during trial run period.

	Control	Test Station
pH	6.6*	5.7**
Alkalinity (ppm)	8	0
Acidity (ppm)	3	6
Sulphate (ppm)	12	23
Specific Conductance (micromhos @ 25C)	39	73

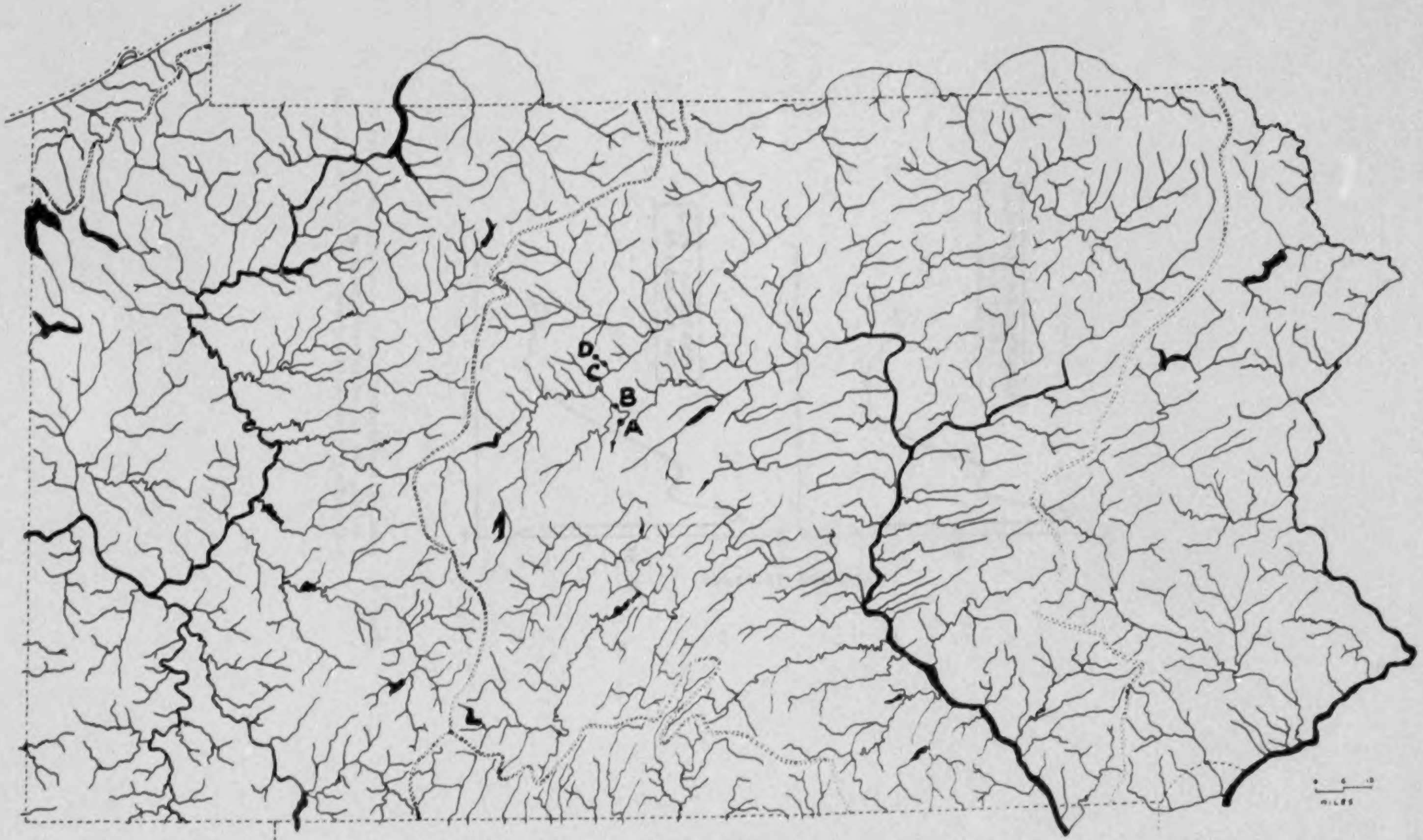
*19 May - 6.4
**19 May - 4.9

Table 4. Water quality in Upper Three Runs 29 April 1970 and during trial run period.

	Control	Acid Tributary	Test Station
pH	6.7*	3.3	5.0**
Alkalinity (ppm)	2	0	0
Acidity (ppm)	2	100	6
Sulphate (ppm)	8	320	28
Specific Conductance (micromhos @ 25C)	36	870	88

*18 May to 25 May - 6.43
**18 May - 5.0; 22 May - 5.5; 25 May - 5.5; 27 May - 4.5 (flood water)

Figure 8. Map of Streams Used in Field Studies



A. Control Site - Black Moshannon Creek
B. Test Site - Black Moshannon Creek
C. Control Site - Upper Three Runs
D. Test Site - Upper Three Runs

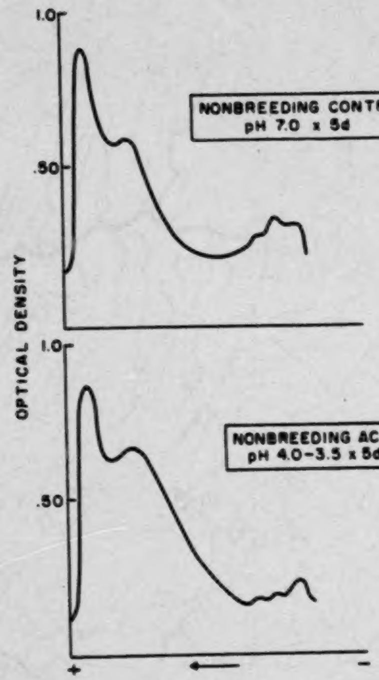


Figure 9. Hemoglobin microdensitometric tracings (scan speed - 1 cm/min, 540 mμ) of polyacrylamide gels from nonbreeding, acute acid exposed trout (pH 4.0-3.5 x 5d).

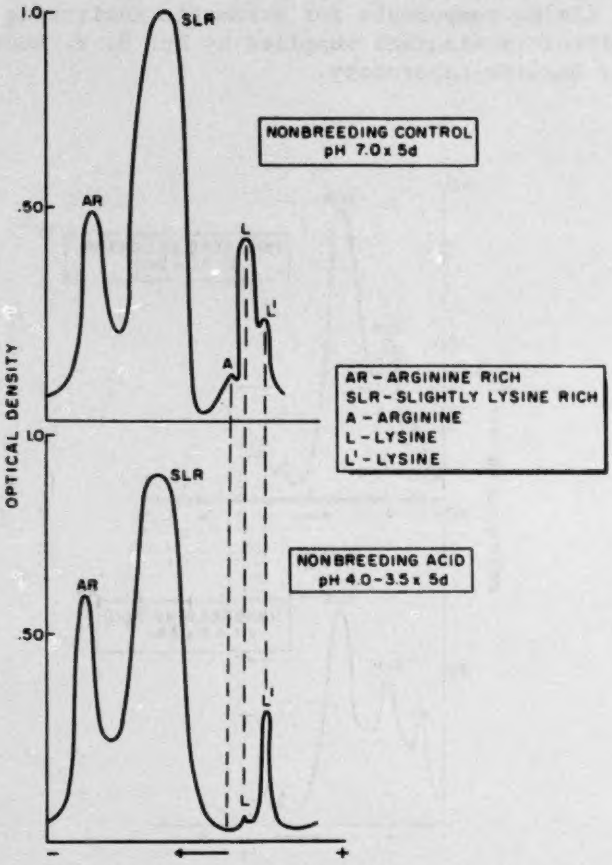


Figure 10. Total blood histone microdensitometric tracings (scan speed - 1 cm/min, 660 mμ) from polyacrylamide gels from nonbreeding, acute acid exposed trout (pH 4.0-3.5 x 5d).

of extract is loaded on the gel. Only two major peaks (AR and SLR) are resolvable. Figure 11 shows that better resolution is obtained with a lesser concentration of histone extract. In this instance, two SLR fractions are separated. However, if one is interested in quantifying changes in minor fractions it is preferable to use higher concentrations of extract.

The final figure 12 shows the Mount-Brungs gravity flow system used in our studies. This was equipped with siphons, solenoids, pH meter and related timing components for automatic monitoring of pH following circuitry diagrams supplied by Dr. R. W. Andrews of the Duluth Water Quality Laboratory.

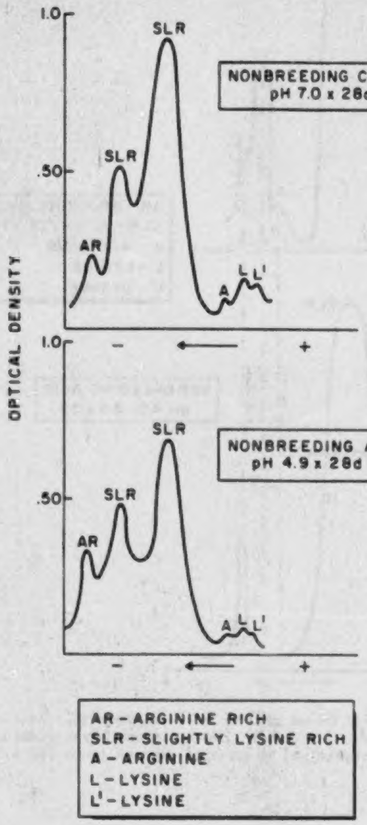


Figure 11. Total blood histone microdensitometric tracings (scan speed - 1 cm/min, 660 mμ) of polyacrylamide gels from nonbreeding, acid exposed trout (pH 4.9 x 26-28d).

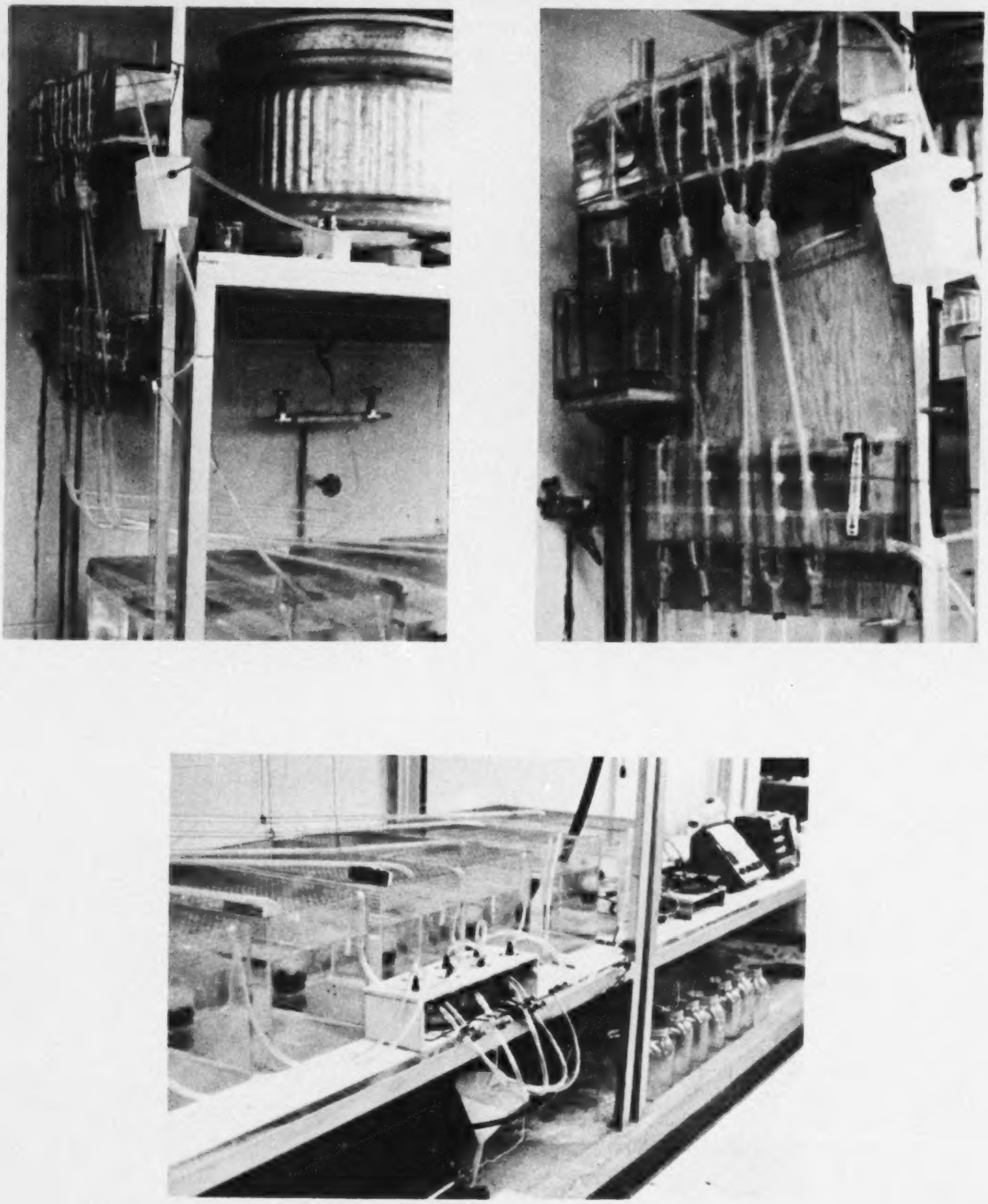


Figure 12. Mount-Brungs Type Gravity Flow Diluter System

*U.S. GOVERNMENT PRINTING OFFICE: 1972 484-482/35 1-3

1 Accession Number
W

2 Subject Field & Group

SELECTED WATER RESOURCES ABSTRACTS
INPUT TRANSACTION FORM

5 Organization
Pennsylvania State University
Department of Biology
University Park, Pennsylvania 16802

6 Title
HISTOCHEMICAL AND CYTOPHOTOMETRIC ASSAY OF ACID STRESS IN FRESHWATER FISH

10 Author(s)
Anthony, Adam
Cooper, Edwin L.
Mitchell, Robert B.
Neff, William H.
Therrien, Chester D.

16 Project Designation
FWPCA, Grant No. 18050DXJ

21 Note

22 Citation

23 Descriptors (Starred First)
*Fish Physiology, *Bioassay, *Acidic Water, *Water Pollution, Pennsylvania, Analytical Techniques, Microscopy, Bioindicators

25 Identifiers (Starred First)
*Fish Histochemistry, *Analytical Histochemistry, *Microspectrophotometry, Cytophotometry, Microspectroscopy

27 Abstract
The feasibility of using histochemical and histopathological changes in brook trout, longnose dace and fathead minnows as bioindicators of acid pollution was investigated. Laboratory studies entailed using a gravity flow diluter system. Field studies involved using net traps in polluted streams. Exposure durations were 4-5 days and 28-30 days. Histochemical and cytophotometric analyses were made of gills, Stannius corpuscle, blood, spleen, kidney and liver.

The primary mode of acid toxicant action is gill damage which results in impaired respiratory, excretory and liver functions. Short term indices of acid stress include: colloidal iron and PAS staining of gills and renal Stannius corpuscles. A useful bioindicator of prolonged acid exposure is decreased azure B-RNA staining of liver cells; this assesses the extent of liver impairment and reflects a reduced tolerance of fish to other toxicants.

Sublethal levels of acidity are not cumulative. However, pH levels of about 5.0 should be considered hazardous since they prove toxic to breeding fish having increased oxygen needs and also reduce the liver's ability to detoxify noxious substances present in acid polluted waters.

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WR:102 (REV. JULY 1969)
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